DETERMINATION OF ALCOHOL ETHOXYLATES IN ENVIRONMENTAL SAMPLES USING DERIVATISATION AND LC/MS

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ABSTRACT

A new method for the analysis of alcohol ethoxylates (AEs) using liquid chromatography with electrospray ionisation mass spectrometry (LC/ESI-MS) is described. The procedure incorporates a novel derivatisation step with phthalic anhydride for the analysis of $EO_{0\text{-}20}$ ethoxylates in a single analysis. The derivatives obtained have proved to be very stable and the negative ion spectra show reduced background ions and competing adduct formation as compared to positive ion spectra. An automated solid phase extraction (SPE) step is used to allow both pre-concentration and clean-up of the environmental samples. The addition of 40 % v/v methanol prior to loading samples provides more efficient recovery of AEs across the C_{12} to C_{18} range than previously reported in the literature, reducing hydrophobic losses. Recoveries from final effluent spiked at 100 μg/L total AE, for the 126 species analysed, were found to be in the range 55 – 117%, with approximately 100 of the individual analytes having recoveries of 90 –105 %. A method detection limit of 0.02 μg/L for individual ethoxylate components is reported with the instrument operated in scan mode over the range m/z 300 to 1300. The method was applied to sewage effluent and influent samples, with AEs determined at approximately 7 and 5000 μg/L, respectively, indicating efficient removal of AEs in the sewage treatment plant. The AEs in the final effluent consisted primarily of fatty alcohols (FAs). Similar optimisation in the extraction of sediment and sludge samples was carried out with ultrasonic, Soxhlet and accelerated solvent extraction (ASE) methodology being compared. The most efficient technique was ASE, which was then used to analyse an activated sludge sample, where again significantly high levels of FAs, compared to other AEs, were found in the sample. This data and the final effluent data are consistent with other current monitoring data for AEs and show the importance of a method capable of extracting and ionising the free alcohol efficiently. The AE fingerprint during biodegradation studies was also accurately determined giving elucidation of mechanisms of primary degradation. Linear and branched AEs were investigated with metabolite identification also carried out for the linear AE. Biodegradation of native AEs present in sewage influent was also studied in direct discharge scenarios to aid in risk assessment in situations where sewage treatment is absent.

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GLOSSARY

ASE	Accelerated solvent extraction
ACN	Acetonitrile
AES	Alcohol ether sulphate
AES	Alcohol ethoxylated sulphates
AEs	Alcohol ethoxylates
ABSs	Alkyl benzene sulphonates
APEs	Alkylphenol ethoxylates
ASMS	American Society for Mass Spectrometry
AISE	Association International de la Savonnerie, de la Détergence des Produits d' Entretien
APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photoionisation
BOD	Biological oxygen demand
CE	Capillary electrophoresis
CAE	Carboxylated AEs
COD	Chemical oxygen demand
CESIO	Comité Européen des Agents de Surface et leurs Intermédiaires Organiques
CAS	Continuous activated sludge
CMC	Critical micelle concentration
DCM	Dichloromethane
DC	Direct current
DLI	Direct liquid introduction
DO	Dissolved oxygen
DTDMAC	Ditallow dimethyl ammonium chloride
EC_{50}	50 % Effective concentration
ESI	Electrospray ionisation
ERASM	Environmental Risk Assessment and Management committee
EO	Ethylene oxide
EICs	Extracted ion chromatograms
FAES	Fatty alkyl ether sulphate

FAS	Fatty alkyl sulphate
FID	Flame ionisation detection
FIA	Flow injection analysis
FTICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
GCB	Graphitised carbon black
HFBA	Heptafluorobutyric acid
HPLC	High performance liquid chromatography
HLB	Hydrophile-lipophile balance
i.d.	inner diameter
IC	Inorganic carbon
ISO	International Standards Organisation
K_{ow}	Octanol-water partition coefficient
LC_{50}	50 % Lethal concentration
LOD	Limit of detection
LOQ	Limit of quantification
LAS	Linear alkylbenzene sulphonates
LC	Liquid chromatography
LC/MS	Liquid chromatography/mass spectrometry
LLE	Liquid-liquid extraction
MALDI	Matrix assisted laser desorption ionisation
MSPD	Matrix solid phase dispersion
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
MDL	Method detection limit
MTBE	Methyl t-butyl ether
MBAS	Methylene blue active substances
MBI	Moving belt interface
MRM	Multiple-reaction monitoring
1-NC	1-Naphthoyl chloride
NOEC	No observed effect concentration
NP	Nonylphenol
NPEs	Nonylphenol ethoxylates

NMR	Nuclear magnetic resonance spectroscopy
ODS	Octadecylsilica
PAHs	Polyaromatic hydrocarbons
PEG	Polyethylene glycol
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration
PLE	Pressurised liquid extraction
Q	Quadrupole
Q-TOF	Quadrupole-time-of-flight
QC	Quality control
RF	Radio frequency
RPLC	Reversed–phase liquid chromatography
STW	Sewage treatment works
SIM	Single ion monitoring
S/N	Signal to noise
SPE	Solid phase extraction
SPME	Solid phase microextraction
TBAH	Tetrabutylammonium hydroxide
TEAH	Tetraethylammonium hydroxide
THF	Tetrahydrofuran
TPS	Tetrapropylenebenzene sulphonate
TSP	Thermospray
TOF	Time-of-flight
TICs	Total ion chromatograms
TFA	Trifluoroacetic acid
UV	Ultra violet
WWTPs	Wastewater treatment plants

UNITS AND NOTATION

1 bar = 10^5 Pa

1 atmosphere (atm) = 101325 Pa

1 torr (= 1 mmHg) = 133.325 Pa

Pound/in.^{2} (psi) = 6894.76 Pa

1 atm = 760 Torr = 760 mmHg

1 molar = 1 M = 1 mol L^{-1}

Prefixes

p	n	μ	m	c	d	k	M	G
pico	nano	micro	milli	centi	deci	kilo	mega	giga
10^{-12}	10^{-9}	10^{-6}	10^{-3}	10^{-2}	10^{-1}	10^{3}	10^{6}	10^{9}

Chapter 1

INTRODUCTION: SURFACTANTS

1.1 History

The oldest man-made surfactant is soap. A tablet from the Sumerians shows that they had already produced soap in 2500 B.C. This tablet is noteworthy for two reasons; firstly it documents the first known chemical reaction, detailing the quantity of oil and wood ash that have to be mixed and heated and, secondly, it contains the only record from the pre-Christian era on the use of soap for washing textiles [1].

The rapid development of the chemical industry and increasing use of surface-active compounds for technical and domestic applications, led to the first commercial surfactant based entirely on petrochemical feedstocks in the early 1930s [2]. These anionic branched alkyl benzene sulphonates (ABSs) rapidly became the most used surfactant in the USA and the rest of the world where advanced chemical technology allowed their production. The replacement of soap in laundry products was essentially driven by two factors: the undesired formation of insoluble calcium or magnesium salts, which precipitated on the clothes and the much cheaper production costs of ABS.

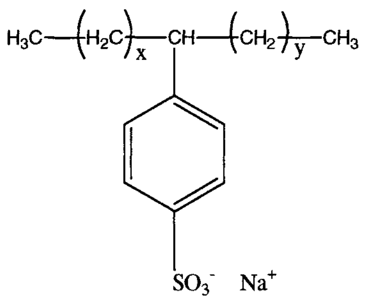
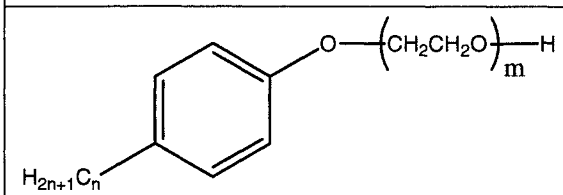
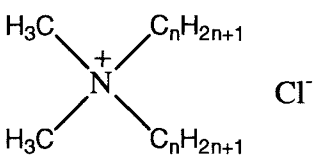
However this widespread use of ABS led to the unexpected problem of foaming in sewage works and river water. This observation was a consequence of physical properties that had originally been responsible for its success [2]. Shortly after the described environmental problems it was recognised that the tetrapropylene starting materials led to a branched hydrophobic moiety which was resistant to biodegradation. This eventually led to the development of linear alkylbenzene sulphonates (LAS), profoundly enhancing biodegradability and hence reducing the overall levels of surfactants found in the environment.

1.2 General properties and types of surfactants

The term "surfactant is derived from "surface active agent". This type of chemical incorporates both hydrophobic and hydrophilic character into their structure. The most common structure being a linear fatty type molecule with one end that is oil soluble and one end water soluble. Of practical interest is the interface between aqueous solutions and soil particles on clothing or the interface between two liquids such as water and oil. Applied correctly, a surfactant can emulsify otherwise immiscible liquids. In industrial terminology, the word detergent is synonymous with surfactant [3]. More precisely, however, detergents are a complex mixture of various ingredients such as surfactants, builders (e.g. phosphates or other suitable chelants) and bleaches [4]. Formulations are balanced to achieve the required performance characteristics.

Commercial surfactants are classified as "anionic", "cationic" or "nonionic" based on the nature of their ionic charges in solution. This classification applies to the hydrophilic end of the molecule, as the hydrophobic end is always nonionic. Therefore "anionic" refers to negatively charged surfactants, "cationic" refers to positively charged surfactants, while "nonionic" refers to those that are uncharged in solution. Table 1.1 shows the chemical structure of typical surfactants of each type. Anionic surfactants constitute the most important group in terms of consumption on a worldwide basis. The nonionics are a very important group of products with the majority consisting of adducts of long-chain alcohols or alkylphenols with a number of ethylene oxide (EO) units. The most representative cationic surfactants are quaternary ammonium derivatives in which the N atom is bonded to four alkyl groups. Cationics are applied as bactericides and disinfectants but mainly used as fabric conditioners where they adhere to the surface of clothes.

Table 1.1 Chemical structures of common surfactants

Structure	Chemical name
ANIONIC	
 x+y: 7-10	Linear alkyl benzene sulphonate (LAS)
C_nH_{2n+1}-O-$(CH_2CH_2O)_m$-$SO_3^-Na^+$ n: 12-16; m: 0-12	Alcohol ethoxy sulphate (AES)
R-COO^-Na^+; R: C_{11} to C_{17}	Soap
NONIONIC	
 n: 8,9; m: 1-20	Alkylphenol ethoxylates (APEs)
C_nH_{2n+1}-O-$(CH_2CH_2O)_m$-H n: 12-18; m: 0-20, usually averaging 7 -12 for domestic detergents	Alcohol ethoxylates (AEs)
CATIONIC	
	Ditallow dimethyl ammonium chloride (DTDMAC)

1.3 *Production and use of surfactants*

The world surfactants consumption for 2003 is summarised in Table 1.2. This data was taken from Comité Européen des Agents de Surface et leurs Intermédiaires Organiques (CESIO) statistics [5] which were presented at the 6^{th} World Surfactants Congress. As can be seen there were 18.2 million tons of surfactants consumed, of which 9.0 million tons was soap. Soap is considered as a natural anionic surfactant and because of its widespread use is not always considered in statistical studies of surfactants. If we consider non-soap surfactants, one of the major uses is household detergents. These account for 40 % of the end-user application [5]. Just three global companies together dominate 62 % of the market (P&G – 29, Unilever – 22 and Henkel 11 %) [5]. CESIO statistics for Western Europe are shown in Table 1.3 [6]. These indicate a total surfactant production of 2.6 million tons in 2002. The major categories of this total surfactant production are shown in Table 1.3. This indicates that nonionics are the most used surfactant class with 1279 x 10^3 tons being produced in 2002, of which 803 x 10^3 tons were alcohol ethoxylates (AEs), the most produced surfactant type in Western Europe.

In terms of worldwide consumption anionic surfactants have the largest market share and are used especially in household detergent applications. From the anionics listed in Table 1.2, LAS has an annual global consumption of 4.5 x 10^6 tons and has a wide application because of its excellent detergency properties and cost-performance ratio [2]. Nonionic surfactants are important because of their capacity to remove oily soil from fabric [7]. They are also less sensitive to water hardness and effective at relatively low concentrations compared to anionics [7]. The APEs derived from nonylphenol (NP) comprise 80 % of the market volume with the other being octyl phenol derived [2]. The persistence and toxicity of some of the biodegradation intermediates of APEs has led to reduced use in several countries. However the cheap cost of production coupled with good performance has hampered their total replacement by environmentally acceptable alternatives. AEs have become increasingly important in recent years, due to efforts to replace APEs. This fact is reflected in the data in Table 1.3, where AEs are consumed at a volume approximately ten times greater than APEs in Western Europe.

Table 1.2 World surfactants consumption 2003 [5]

Surfactant type	Million tons	
Soap	9.0	
Anionics	4.5	
Linear alkylbenzene sulphonate (LAS)		2.9
Branched alkylbenzene sulphonate		0.2
Fatty alkyl ether sulphate (FAES)		0.8
Fatty alkyl sulphate (FAS)		0.6
Nonionics	1.7	
Alcohol ethoxylates		1.1
Nonylphenol ethoxylates (NPE)		0.6
Quats	0.5	
Amphoterics	0.1	
Others	2.4	
Total	**18.2**	

Surfactants can be produced from both petrochemical and oleochemical feedstocks. Crude oil and natural gas make up the petrochemical class. Palm oil, palm kernel oil, tallow and coconut oil are the most used oleochemical or renewable resources. Surfactant products account for only 1.5 % of petrochemical use [2]. The main starting materials comprise of ethylene, n-paraffins and benzene which in turn are converted to α-olefins, oxo-alcohols, primary alcohols, ethylene oxide and alkyl benzenes and then further modified to yield the desired surfactants.

The worldwide capacity of basic oleochemicals, fatty acids and fatty alcohols has been estimated at 5.2 x 10^6 tons [8]. The most important C_{12}/C_{14} alkyl derivatives are found in coconut and palm kernel oil. The C_{16} and C_{18} homologues are found in palm oil and tallow.

Table 1.3 Surfactant consumption in Western Europe during 2002 [6].

Surfactants	**Production (10^3 tons)**	**Sales and captive use (10^3 tons)**
Anionics		
LAS	408	249
Alkane sulphonates	100	88
Alcohol sulphates	86	68
Alcohol ether sulphates	331	274
Other anionics	76	64
Total anionics	1001	743
Nonionics		
AEs	803	695
APEs	83	59
Other ethoxylates	185	152
Amine oxides	10	9
Other nonionics	198	186
Total nonionics	1279	1101
Cationics		
Total Cationics	234	239
Amphoterics		
Total amphoterics	76	73
Total surfactants	**2590**	**2156**

There is a shift from petrochemically-based to oleochemically-based surfactants, especially in the alcohol derivatives field [2]. This is explained by some of the following reasons [8]:

- Perceived better mildness from natural products
- Good ecotoxicology profiles

- World class cost base with secure access to competitive raw materials

In the period from 1994 to 1997 there was a 17 % reduction in growth of the traditional petrochemical surfactants like alkylphenol ethoxylates [8]. This can be explained in part by the concern over environmental safety of nonyl phenol ethoxylates. In reality there is also an increasing demand for surfactants having the diverse and effect-specific nature obtained from oleochemical origin [8].

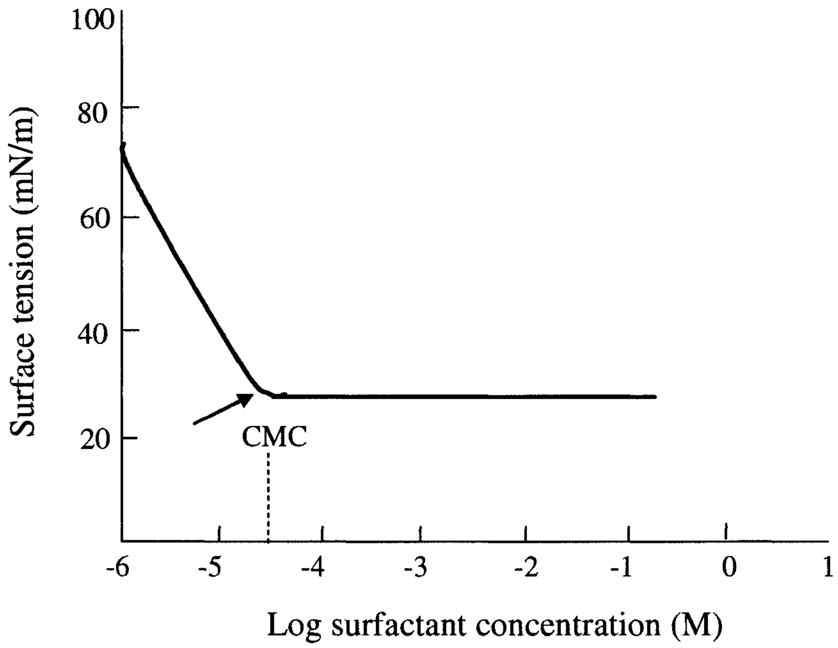
1.4 Physical-chemical properties

Some of the properties which give surfactants their specialist activities have implications for their fate and behaviour in the environment. Properties in aqueous solution and at interfaces are of particular interest in the context of this thesis, where behaviour in waters, sediments and soils is important. Characteristic properties of surfactants include surface tension, micellation, hydrophile-lipophile balance (HLB), cloud point and foaming.

1.4.1 SURFACE TENSION AND MICELLATION

In highly dilute aqueous solutions surfactants exist in monodisperse form and are concentrated at the interface by hydrophilic-hydrophobic oriented absorption [7]. At the air-water interface surfactants accumulate with their hydrophobic tail oriented towards the air and the hydrophilic part towards the aqueous environment. This leads to a reduction in surface tension of the liquid and surfactants owe their name to this property. Once the surface is completely covered, a point of inflection is reached where increasing the surfactant concentration further has little impact on the surface boundary and the molecules start to form aggregates. This point is called the critical micelle concentration (CMC). This phenomenon is demonstrated in Figure 1.1, adapted from Holt et al. [9]. Typical CMC values for AEs are in the range 10^{-3} to 10^{-5} M [9].

Figure 1.1 Surface tension of surfactant solutions



1.4.2 ADSORPTION AND WETTING

Adsorption and wetting are based on the property of surfactants, which causes them to accumulate at solid/liquid interfaces. This leads to technical applications such as detergency [7,10]. This adsorption is related to the surface properties of the solid and also the structure of the hydrophile and hydrophobe. Mechanisms by which nonionic surfactants absorb include hydrogen bonding and Van der Waals dispersion forces [7]. This property, however, provides major problems in the handling and analysis of surfactants in trace environmental analysis. Preparation of glassware and equipment requires special attention as the possibilities of contamination are many [9].

1.4.3 HYDROPHILE-LIPOPHILE BALANCE AND CLOUD POINT

The hydrophile-lipophile balance (HLB) is a measure of the emulsifying and solubilising character of a nonionic surfactant [10]. It provides an expression of the relationship of the size and strength of the polar and nonpolar groups of a surfactant. The HLB for an AE can be calculated from Equation 1.1 [10], where E is the weight % of the ethylene oxide content in the nonionic surfactant.

$$HLB = E/5 \qquad \text{Equation 1.1}$$

For example an HLB value of 10-15, where the EO content is 50-75 % w/w suggests the application may be that of a detergent [7].

The cloud point is a particularly important physical property of a surfactant because it determines the optimum conditions for detergency. It is the temperature at which the nonionic surfactant oils out of solution due to addition of heat [10]. It is defined technically as the temperature at which a warm cloudy solution becomes clear on cooling. Generally nonionic surfactants exhibit optimum effectiveness when used a temperatures near their cloud points.

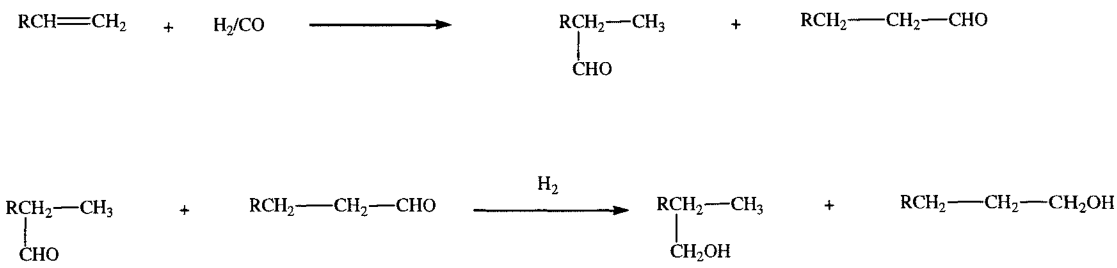
1.4.4 FOAMING

Surfactants accumulate at the interface of a gas bubble in aqueous solution, with their hydrophobic residues directed to the interior of the bubble [7]. The combination of numerous bubbles constitutes foam.

1.5 Alcohol ethoxylates

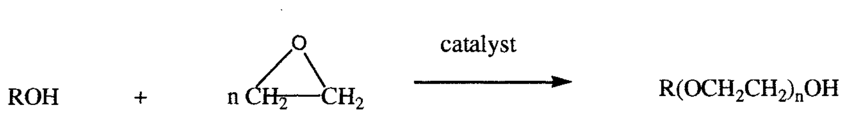
This thesis is concerned with the analysis of alcohol ethoxylates (AEs), which are nonionic surfactants. Their importance in the surfactants industry has already been shown in the preceding sections. AEs are synthesised industrially by base catalysed addition of ethylene oxide to aliphatic alcohols from oleochemical and petrochemical sources. The oleochemical derived alcohols are linear and primary. They contain only an even number of carbon atoms in the homologous chains, between C_{12} to C_{18}. The petrochemical mixtures contain even and odd numbers of carbon atoms in the homologues and are either linear or branched depending on the composition of the olefin feedstock. These so called oxo-AEs from linear olefins are primarily linear but contain also a degree of 2-alkyl substitution and typically contain 11 to 15 carbon atoms. The oxo process is shown in Figure 1.2 [10]. The addition of hydrogen and carbon monoxide to an olefin is carried out using a cobalt catalyst at high temperatures and pressures.

Figure 1.2 The oxo process used in the manufacture of alcohols



The alcohols are then reacted with ethylene oxide in industrial processes resulting in a Poisson-like ethoxymeric distribution of each AE homologue. This reaction is conventionally base catalysed with potassium hydroxide (Figure 1.3) [10]:

Figure 1.3 The reaction of alcohol with ethylene oxide



A typical product distribution from the base catalysed procedure is shown in Figure 1.4. The general structure of AEs analysed in work described in this thesis is represented in Figure 1.5. A commonly used abbreviation of AE structure is $C_n\,EO_m$, where n and m denote the number of carbon atoms (C) in the alkyl chain and ethoxylate groups (EO), respectively.

Household laundry detergents are the single largest end use for AEs, with linear primary AEs being preferred due to their rapid biodegradability [11]. This widespread use and disposal down the drain has led to a need to monitor levels of these surfactants in the environment, a responsibility which has been co-ordinated by the Environmental Risk Assessment and Management committee (ERASM) on behalf of a number of detergent industries and raw materials producers.

Figure 1.4 Typical product distribution of dodecanol reacted with ethylene oxide using KOH as a catalyst.
Adapted from [10].

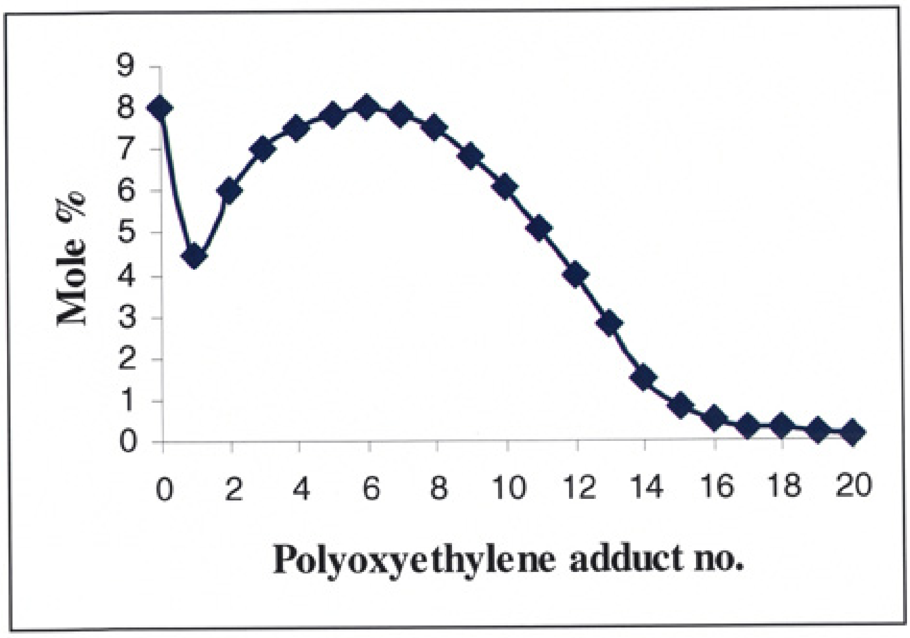
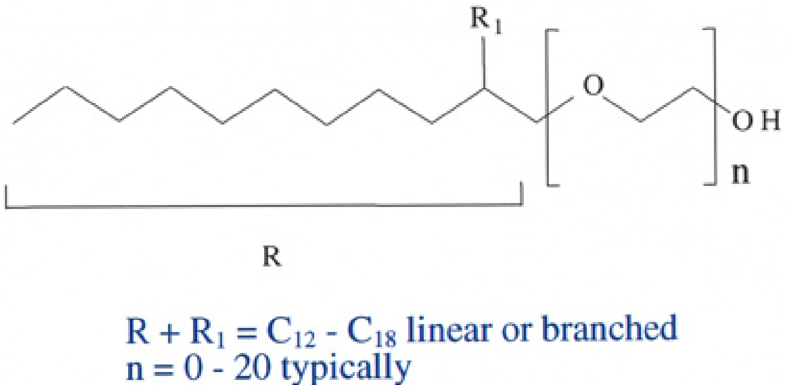


Figure 1.5 Typical structure of alcohol ethoxylates



$R + R_1 = C_{12} - C_{18}$ linear or branched
$n = 0 - 20$ typically
$R_1 = H$, methyl, ethyl etc

1.6 Conclusion

This chapter has discussed the structures, properties, manufacture and application of surfactants. A detailed discussion of the environmental safety and risk assessment of surfactants, with particular reference to alcohol ethoxylates will be included in Chapter 2.

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Chapter 2

ENVIRONMENTAL RISK ASSESSMENT

2.1 General introduction

Surfactants are a major group of chemicals of environmental concern. This is because they are major ingredients in a high volume product category which after use go down the drain and hence have the potential to enter the aquatic and terrestrial environment. Surfactants have relatively high aquatic toxicity, which has made them the focus of environmental compatibility studies for some time [1]. In 1991 the Environmental Risk Assessment of Surfactants Management (ERASM) was created as a joint platform of the European detergent and surfactants producers, the Association International de la Savonnerie, de la Détergence des Produits d' Entretien (AISE) and Comité Européen des Agents de Surface et leurs Intermédiaires Organiques (CESIO). The combined knowledge, expertise and resources of the surfactant producing companies enabled the multi-dimensional requirements for a sound risk assessment, i.e. basic fate and effects data, calculation models, analytical methods for monitoring programmes, to be carried out more effectively [1]. Many of the ERASM activities are dependent on the availability of specific and sufficiently sensitive analytical methods. The needs for analytical support are broad and imply the development of methods for detection and quantification of individual surfactants or surfactant groups in river water, test solutions, sludges, sediments and even in the tissues of aquatic organisms. A task force recruited from analytical experts of ERASM members is responsible for this contribution

2.2 Fate of surfactants and environmental issues

Historically, problems related to surfactants can be considered as follows [2]:

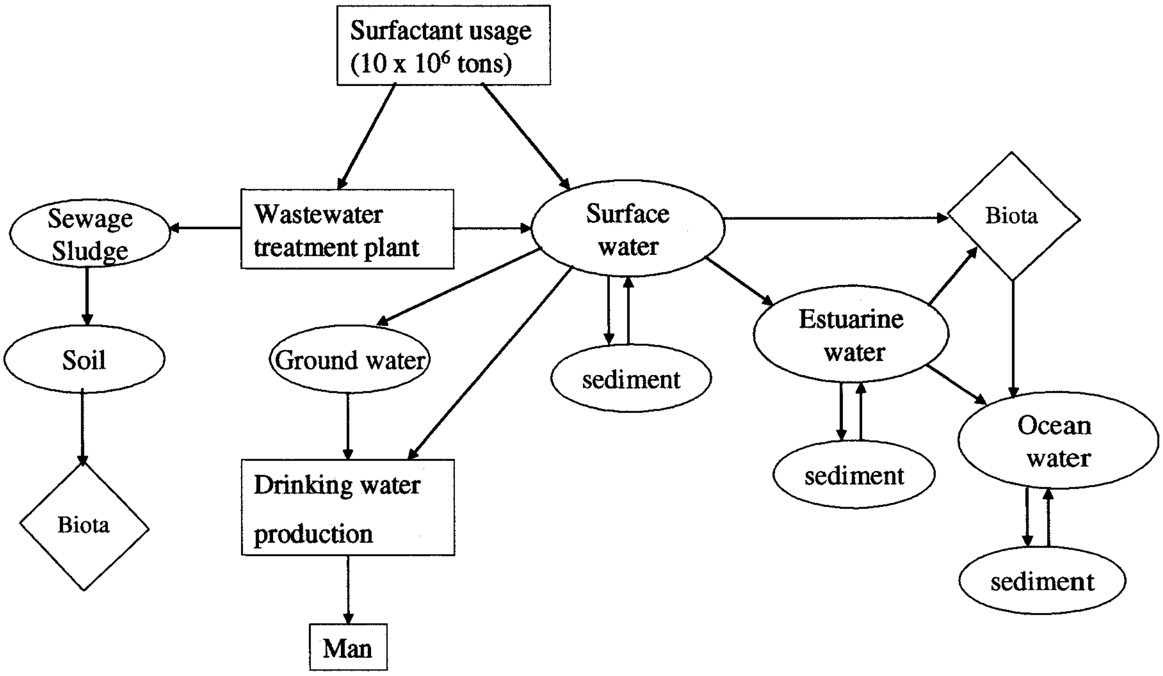
- Foaming in natural and wastewaters produced by poorly biodegradable surfactants of the highly branched alkyl benzene sulphonates (ABS).
- Eutrophication of natural waters due to the high phosphate component of detergents.
- Metabolites of surfactants being more toxic than the parent material, requiring monitoring in wastewater effluents and sewage sludges.

The surfactant industry nowadays attempts to avoid the occurrence of the above issues by means of preventive action.

Awareness of the environmental problems caused by surfactants has led to a series of changes in the surfactant market since the introduction of first generation synthetic surfactants in the 1950s [3]. In the beginning application and economical aspects governed their production but later on their fate after use also had to be taken into account. The fate of surfactants in wastewater treatment plants (WWTPs) is shown in Figure 1.1.

WWTPs provide a primary treatment, which is simply the removal of solid material by mechanical means. Secondary treatments are accomplished by an activated sludge process or trickling filter [4]. In this process the organic components of the wastewater are subjected to aerobic degradation by microbial biocoenosis [3]. Surfactants can be metabolised by adapted micro-organisms using them as energy sources. The initial step yields change in the molecular structure and associated loss of surface-active properties- primary degradation. Ideally complete metabolism of the compound (ultimate degradation), yielding carbon dioxide, water, inorganic substances and bacterial biomass occurs. Sometimes however this end-point is not reached due to insufficient retention in the WWTP, a shock load emitted into the WWTP or slow degradation kinetics of the parent compound and intermediates. This results in surface water receiving trace amounts of surfactant and its metabolites.

Figure 2.1 Fate of surfactants in the environment after discharge into sewers



2.3 Definition of ecotoxicology

Toxicology is the study of the effects of poisonous substances on living organisms. Above a certain concentration, the toxicant has detrimental effects on some biological function. The poisonous substance being present due to pollution which is defined as "the introduction by man into the environment of substances or energy liable to cause hazards to human health, harm to living organisms and ecological systems, damage to structures or amenity, or interferences with the legitimate uses of the environment"[5]. Pollutants can effect living organisms in two ways, firstly by being directly toxic and secondly by causing an adverse change in the organisms habitat which then adversely effects the organism.

It follows from the above definitions that ecotoxicology is not just concerned with the effects of toxicants on one species but on a wide range of interacting species present in an ecosystem [6]. The term ecosystem can be defined as follows: No one population exists alone and populations of different species existing in an area form a community. The term environment denotes all of an individual organism's surroundings, both inanimate such as air, soil and water and other plants and animals and members of its own species. The inanimate surroundings are sometimes called the external environment. Finally, a community and its external environment constitutes an ecosystem and it is the effects of pollutants on ecosystems that form the subject of ecotoxicology [6].

2.4 Risk assessment of surfactants

In the third of a series of workshops, organised by AIS and CESIO, experience gained in the environmental risk assessment of four major surfactants used in laundry and cleaning products was reviewed [6]. The surfactants studied were linear alkyl benzene sulphonate (LAS), alcohol ethoxylates (AEs), alcohol ethoxylated sulphates (AES) and soap. In this work a tiered approach had been adopted, where risk is determined by comparison of the predicted no effect concentration (PNEC) to organisms in ecosystems with the predicted environmental concentration (PEC). The detailed results of the environmental monitoring and derivation of PECs are described in papers by Matthijs et al. [7] and Feijtel et al. [8]. The derivation of PNECs and the risk characterisation are described by Van de Plassche et al. [9]. Detail of how the PECs and PNECs were obtained will now be discussed.

2.4.1 PNECs

A PNEC is firstly derived based on single-species toxicity data. This is then compared with the no observed effect concentration (NOEC) obtained from multi-species in model ecosystems. A final PNEC is derived from the data. The ecotoxicological data set for LAS, AE and AES contains data for test compounds differing in the number of ethoxylate (EO) groups and/or alkyl chain length [9]. For each group of surfactants the data has been normalised to structures typically present in the environment e.g. for AE this was $C_{13.3}EO_{8.2}$. For AEs a large database of toxicity data is available for several taxonomic groups, including algae, crustaceans, fish and worms. A statistical method is used to derive the PNEC from all this data, which was found to be 110 μg/L [9]. This PNEC was then compared with data from AE field studies, where end-points such as reproduction rates were studied. This gave a NOEC of 42 μg/L, but as the type of AE studied required a big normalisation factor the final PNEC of $C_{13.3}EO_{8.2}$ was confirmed as 110 μg/L [9].

2.4.2 PECs

The PEC is calculated using information or data on [8,10]:

- release,
- in-sewer removal,
- treatment efficiency,
- dilution,
- in-stream removal

For release the following formula (Equation 2.1) has been used [8]:

$$C_{influent} = \frac{X \times 10^6}{Y \times Q \times 365} \text{ (g/L)} \qquad \text{Equation 2.1}$$

Where $C_{influent}$ is the concentration of a detergent chemical in influent waste water (g/L), X is the quantity of detergent marketed (tons/year), Y is the population of the market area and Q is the per capita waste flow rate (L/capita/day). The monitoring data show an in-sewer removal for AEs of about 38 % [10]. This removal is due to adsorption and biodegradation. Models for treatment efficiency from activated sludge WWTPs predict removal of AEs to be 98-99 %, with 1 to 2 % being discharged to receiving surface waters. A dilution factor of 3 and an in-stream removal of 0.7 day^{-1} are also used in the calculations [10].

PEC/PNEC for AE were <0.05 [10], indicating the risk for AE in the aquatic environment is low. Similar data was obtained for LAS and AES, however for soap the ratio was almost 1. This was due to the calculation of the PNEC for soap being based on acute toxicity data only. It is thought however that soap was unlikely to be more toxic than the other surfactants and new data has been generated to prove this [11].

2.5 *Toxicity of alcohol ethoxylates*

The general picture of the mechanism of toxicity of surfactants is that they interfere with membrane processes [12]. This modification in structure may cause harmful or beneficial effects on the activity of various enzymes in biological systems [13]. In general, AEs are generally biodegradable but standard tests with fish and daphnia (water flea) show that they have a relatively high aquatic toxicity [14]. An interesting comparison between AEs and APEs in terms of their environmental acceptability is that the parent molecules of APEs are generally less toxic than AEs but the reverse is true of the biodegradation intermediates [12,15].

In terms of structure related toxicity of AEs it has been shown that increasing hydrophobicity leads to a decrease in algal growth. Branched and linear AEs were compared by Dorn et al. [16]. A 50 % effective concentration (EC_{50}) of 7.5 mg/L was obtained for the branched AE, whereas an EC_{50} of 0.7 mg/L was obtained for the equivalent linear AE.

Similarly several studies have tried to relate AE's toxicity to crustaceans e.g. *Daphnia magna* to their structural characteristics. In one example Wong et al. [17] found that increasing ethoxylate chain length caused a decrease in acute toxicity. Linear AEs have been shown to be more acutely and chronically toxic than branched AEs in *Daphnia magna* toxicity tests, 50 % lethal concentrations (LC_{50}) of 1.3 mg/L for linear $C_{12\text{-}15}EO_9$ and 6.1 mg/L for branched $C_{13}EO_7$ were obtained [16,18].

Like the other test organisms, relationships between toxicity and chemical structure have been indicated for fish. Linear AEs were again more toxic than branched in experiments with fathead minnow [16]. Acute toxicity was shown to increase with increasing alkyl chain length for the same test species in the work of Wong et al. [17].

Overall the results indicate that the more hydrophobic AEs are generally more toxic than the less hydrophobic ones [13]. Toxicity increases with an increase in alkyl chain length and decreases with an increase in ethoxylate chain and decreases with methyl branching in the alkyl chain [19]. Surfactant liposolubility appears to be a major factor in determining toxicity. Ideally speaking the safety assessment of a nonionic surfactant should be based on a complete set of ecological data, including ultimate biodegradability, fish-, daphnia-, and algal-toxicity. Only a complete data set for the raw material gives the manufacturer the certainty that the formulation will not be classified as dangerous to the environment [14].

2.6 Biodegradation

Biodegradability of surfactants essentially only became an issue as the surfactant industry developed. Tetrapropylenebenzene sulphonate (TPS) or ABS was introduced into detergents in the USA in 1946 [20]. It showed excellent performance and was easily and cheaply produced. A disadvantage of the surfactant became apparent as large mountains of foam were observed on rivers, especially during dry summers and near weirs (e.g. in Germany in 1959 and 1960 [20]). This was due to the fact that TPS was insufficiently biodegradable, leading to laws in Germany in 1961 being passed requiring detergents to be biodegradable. The same events took place worldwide leading to the development of the biologically soft, easily degradable linear alkylbenzene sulphonate (LAS) in Europe, USA and Japan.

Synthetic surfactants are probably the most studied group of general chemicals in terms of their biodegradability [21]. Biodegradation involves the breakdown of chemical structures through biological means. Bacteria are considered to be the dominant organisms in this process, although other micro-organisms may be responsible. Surfactants contain large supplies of reduced carbon which the bacteria can exploit as a source of energy and growth.

The mechanism of biodegradation of AE has been covered in many books and reviews [12,22]. These all point to structural features that influence the biodegradation. Firstly, if we consider the structure of the hydrophobe or alkyl chain; Swisher [22] states that ultimate biodegradation is most rapid with a linear alkyl chain and that single methyl branches, such as those occurring in linear oxo alcohols exert a minimal effect. In contrast highly branched hydrophobes such as tetrapropylene alcohols retard the process greatly. Other factors include the length of the hydrophile (EO chain). AEs containing more than 20 EOs have been reported to reduce the rate of ultimate biodegradation [12]. The initial point of bacterial attack for AEs can be at any one of three sites [22]:

(i) A central fission mechanism separating the hydrophilic and hydrophobic groups with subsequent independent oxidation,
(ii) Attack at the far end of the hydrophobe,
(iii) Attack at the far end of the hydrophile.

When attack is at the far end of the hydrophobe, the surfactant biodegrades by mechanisms similar to those for aliphatic hydrocarbons [23]. Bacterial enzymes oxidise the terminal carbon atoms in a process known as ω-oxidation, followed by rapid biodegradation via the oxidation cycle known as β-oxidation which shortens the alkyl chain by two carbon units at a time [12]. The alcohol or carboxylic acid produced as a result of a central fission mechanism will also rapidly biodegrade by the β-oxidation mechanism. The attack at the far end of the EO chain (ω-hydrophile) can be oxidative or can proceed via a non-oxidative pathway resulting in loss of ethylene glycol units by hydrolysis.

As to which of these processes dominate for a linear AE has been the subject of much discussion. Earlier work conducted using radiolabelled AEs by Nooi et al. [24], suggests ω/β-oxidation of the alkyl chain as the initial mechanism, however the central fission is an important path for linear primary alcohol ethoxylates [22] and both mechanisms have been found to act simultaneously by Steber et al. [25]. It appears that both mechanisms occur in nature, although the bacterial strains found in domestic sewage tend to favour the hydrophile-hydrophobe scission [18].

More recent studies by Marcomini et al. [26], who investigated the biodegradation of linear, monobranched and multi-branched AE, have utilised LC/MS to identify intermediates and mechanisms of biodegradation.. The linear and monobranched (oxo) AE were found to biodegrade by an initial central cleavage mechanism, based on the formation and oligomeric distribution of polyethylene glycol (PEG). No PEG was detected during the biodegradation of the multi-branched AE, but carboxylated AEs at the polyethoxylic chain were identified, maximising at 3% on a molar basis of initial AE concentration. The data suggests the primary point of attack for the multi-branched AE was by the ω-hydrophile mechanism.

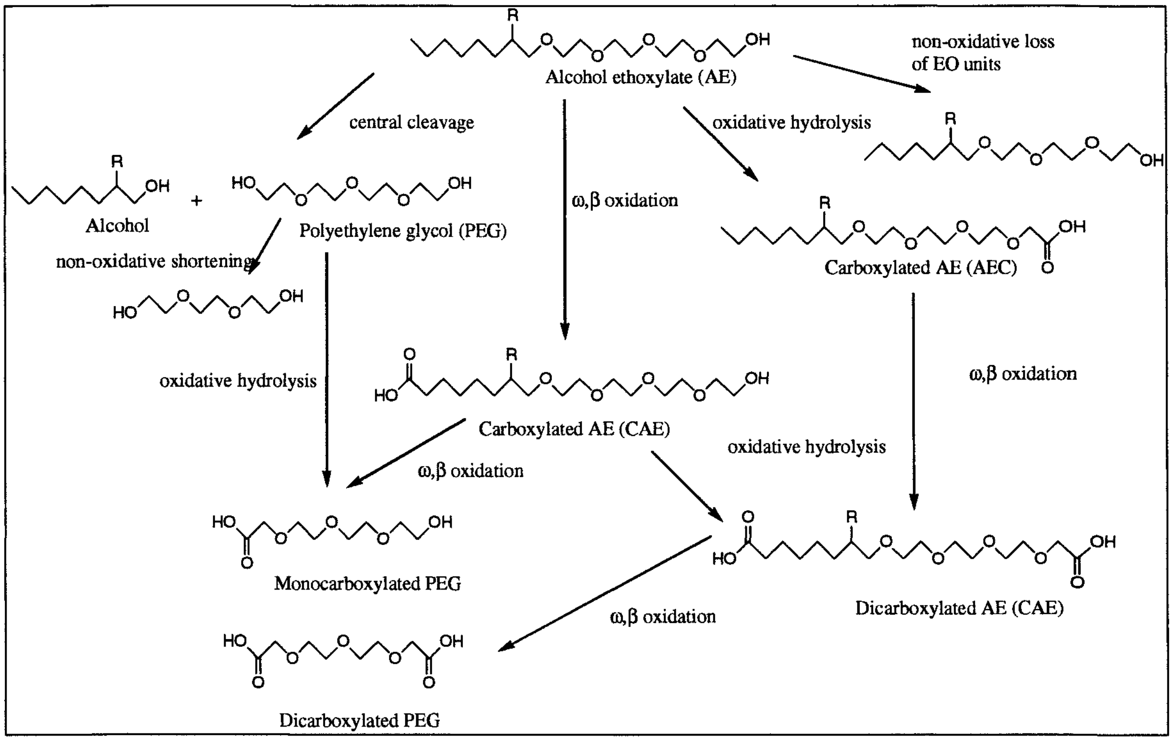
In another piece of work published in the same year Marcomini et al. [27] studied the biodegradation of a 2-butyl-octyl alcohol polyethoxylate. This suggested that the length of side chain could influence the mechanism of attack. Linear and oxo-AE with short 2-alkyl (methyl and ethyl) substituents were found under the same inoculum conditions to undergo a central cleavage mechanism whereas the butyl substituted ethoxylate was found to biodegrade through hydrolytic oxidation of both ethoxylate and alkyl chains. The presence of carboxylic groups on both the hydrophobic and hydrophilic moieties were detected. On a molar basis carboxylated AEs (CAE) were the main metabolites observed (see Figure 2.2), formed through the ω, β-oxidation mechanism. A schematic diagram for some of the proposed mechanisms of AE biodegradation is shown in Figure 2.2 [27].

Di Corcia et al. [28] also investigated a similar butyl branched AE, suggesting that EO chain shortening and to a lesser extent EO chain oxidation were the primary modes of bacterial attack.

2.7 Biodegradation test methods

Terms used in this work include "primary" biodegradation, which means biodegradation of a substrate to an extent sufficient to remove a characteristic property of the original molecule [29]. In its simplest form for a surfactant this can be measured as loss of foaming capacity. A series of test methods for determining "ready" biodegradability are available. Work described in Chapter 9 uses one of these, the International Standards Organisation (ISO) 14593 CO_2 headspace method [30]. The extent to which a chemical is mineralised to CO_2 provides a definite measure of "ultimate" biodegradation [31]. A chemical giving a positive result in this test will undergo rapid and ultimate degradation in the environment. The current Technical Guidance Document [32] recommends that a chemical reaching a pass level of $\geq$ 60% biodegradation is assumed to degrade up to 88% in the environment.

Figure 2.2 Biodegradation pathways proposed in the literature [27]

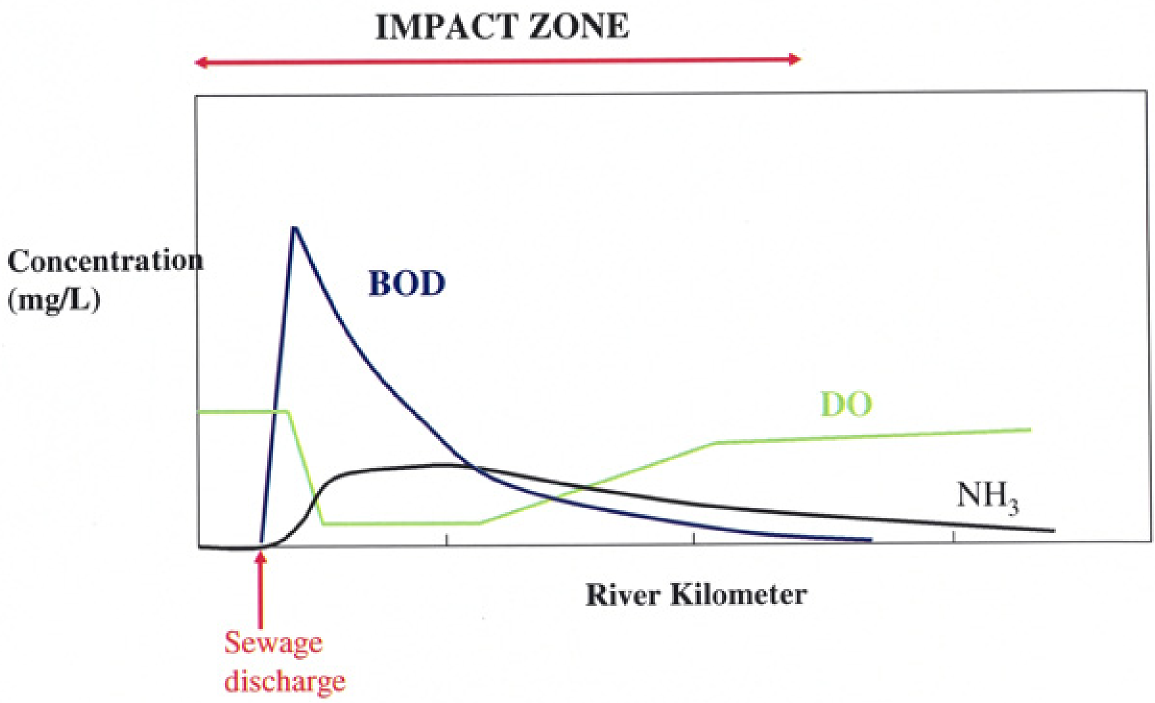


2.8 Direct discharge

In many locations around the world, industrial and domestic waters are disposed directly into the environment with no prior treatment [33]. Currently, extensive biodegradation kinetics are available for major surfactants under conditions of municipal WWTPs [34]. To provide data for biodegradation in river water under direct discharge conditions Peng et al. [33] demonstrated the degradation of methylene blue active substances (MBAS) and LAS was more rapid than chemical oxygen demand (COD). This would indicate that these materials would be at low level once the stream had recovered from the addition of untreated sewage, or degrade as fast as the general organic chemicals present in the wastewater. A model for risk assessing consumer product ingredients in surface waters that receive untreated wastewater was presented by McAvoy et al. [35]. This approach uses an impact zone concept (Figure 2.3), where the receiving water can be thought of as a natural wastewater treatment system. After the river has recovered via self-purification, the ecosystem can be assessed by traditional risk assessment methods.

In Figure 2.3, at the point of sewage discharge the biological oxygen demand (BOD) and ammonia (NH_3) are high and dissolved oxygen (DO) is low. These parameters can be seen to recover to their original values, after the sewage discharge, in a distance known as the impact zone.

Figure 2.3 The impact zone concept [35]



2.9 Conclusion

This chapter has looked generally at the environmental risk assessment of surfactants, with a focus on AEs, especially the biodegradation of these compounds. Chapter 9 studies the biodegradation of highly ethoxylated AEs and also investigates the biodegradation of AEs, at their native levels in a sewage influent, in simulated direct discharge conditions.

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METHODOLOGY FOR THE EXTRACTION AND ANALYSIS OF ALCOHOL ETHOXYLATES

3.1 General overview and history of instrumental techniques

Household laundry detergents are the single largest end use for alcohol ethoxylates (AEs), with linear primary AEs being preferred due to their rapid biodegradability [1]. This widespread use and disposal into the domestic sewage system has led to a need to monitor levels of these surfactants in the environment. This responsibility has been co-ordinated by the Environmental Risk Assessment and Management committee (ERASM) on behalf of a number of detergent industries and raw materials producers. Due to the efficiency of removal of AEs in activated sludge treatment plants (97%) [2] environmentally orientated analytical procedures for the determination of these compounds must be capable of quantitation at concentrations of <10 to 100 μg/L in receiving waters [3]. There is also a need for speciation of the individual homologues and oligomers as toxicity and biodegradation of AEs depend on the length of both the alkyl and polyethoxylate chains [3]. A recent review discussing the occurrence, fate and effect in the aquatic and terrestrial environment of AEs used as adjuvants in pesticide formulations has been conducted by Krogh et al. [4].

Analytical methodology for the analysis of AEs has progressed rapidly over the years. Non-specific spectrophotometric and titration methods have been reviewed by Holt et al. [5]. However, these methods are not suitable for the low concentration of nonionic surfactants found in most surface waters. The lack of a chromophore in the AE molecule means that high performance liquid chromatography (HPLC) using the most commonly used detectors depends on the formation of derivatives amenable to ultra violet (UV) absorption or fluorescence detection. Derivatives used include phenyl isocyanate, which is widely used in environmental analysis to provide a UV chromophore [6]. Derivatisation with 1-naphthoyl chloride (1-NC) and 1-naphthyl isocyanate [7] yield fluorophores, adding more sensitivity and selectivity. The separation of AEs by HPLC methods can be based on ethoxymeric distribution using normal phase HPLC, or as is more commonly used in environmental analysis the separation is based on the resolution of the hydrophobic homologues under reversed phase HPLC conditions. The combination of HPLC with evaporative light scattering detection [8] permits the analysis of AEs without the requirement of derivatisation, but lacks the sensitivity required for environmental analysis.

Application of gas chromatography (GC) has been limited to compounds with less than 5 ethoxy units, due to the high polarity, low volatility and thermal instability associated with the higher oligomers [9]. However biodegradation of AEs has been studied by GC-flame ionisation detection (FID) of the alkyl bromides produced by acid cleavage of the ether linkages with hydrogen bromide [10]. To provide detail of homologues, oligomers and isomers the current methodology has focussed on liquid chromatography/mass spectrometry (LC/MS). LC/MS methods utilising thermospray [11] and electrospray ionisation (ESI) [12,13] have been used to provide such information. However these methods suffer fundamental flaws in that the ionisation efficiency of the individual ethoxylates vary greatly and EO_{0-2} are not normally detected or produce a weak signal. LC/MS methods utilising atmospheric pressure chemical ionisation (APCI) have been successfully employed for the analysis of AEs in environmental samples [14-16]. However APCI was also shown to have low sensitivity for the lower ethoxymers and to give some thermal degradation of higher ethoxymers [17]. Method development has now gone full circle with derivatisation strategies converting alcohols to ionic or solution ionisable compounds to aid the LC/MS process [18]. The work of Dunphy et al. [19] utilised the reaction of the terminal hydroxyl group of each surfactant species with 2-fluoro-N-methylpyridinium *p*-toluenesulphonate (pyridnium reagent), imparting a cationic charge allowing all species including the free alcohol and EO_1 to be determined by ESI-MS.

This thesis details an alternative derivatisation approach for the analysis of AEs by LC/MS, which utilises the reaction of the hydroxyl group with phthalic anhydride. The derivatisation has its origins as a classical wet chemical procedure for the determination of hydroxyl number [20]. Phthalic anhydride derivatives have previously been used as a UV chromophore for HPLC [21] and also capillary electrophoresis (CE) [22,23]. In this thesis phthalic anhydride derivatisation of AEs is used to facilitate detection of all ethoxylates and the free alcohol by negative ion ESI-MS. The derivatisation and LC/MS methodology is linked to solid phase extraction (SPE) for the determination of environmental levels of AE in influent and effluent samples. The SPE procedure has been optimised in an attempt to give better recovery of the more hydrophobic AE species present in samples, particularly $C_{16,18}EO_{0-10}$, which have traditionally been difficult to quantify.

3.2 Sample preparation for liquid samples

3.2.1 INTRODUCTION

Extraction from environmental waters, where risk assessment requires trace levels (µg/L) to be determined, is a critical part of any analytical method. In general these water samples are too dilute and too complex to be analysed by liquid chromatography (LC) without some preliminary sample preparation. Sample preparation generally involves extraction and concentration of the trace organics present, whilst at the same time removing other compounds (clean-up) that could interfere with the chromatographic analysis. Historically surfactants have been concentrated from water using solvent sublation techniques (Section 3.2.3). This methodology was time consuming, typically requiring large volumes of solvent use and moreover recovery of polar analytes using liquid-liquid extraction (LLE) are low because of their relatively high partial solubility in water [24]. Method development in surfactant analysis was made much easier by the introduction of solid phase extraction (SPE), which will be described in more detail in Section 3.2.2.

3.2.2 SOLID PHASE EXTRACTION

Disposable cartridges for SPE have been available for over 25 years but acceptance of the technique was slow especially in the field of environmental analysis where LLE was traditionally the preferred technique [25]. However increased development in SPE occurred in the late 1990's with the improvement in format, automation and the introduction of new phases [25]. There was also pressure to decrease organic solvent usage [25] and as already discussed certain polar analytes cannot be efficiently extracted by LLE because of restrictions in selection of water-immiscible solvents.

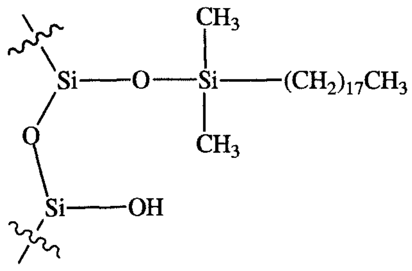
SPE can be used off-line where sample preparation is separated from the chromatographic analysis or on-line where it is directly connected to the chromatographic system. This discussion will mainly focus on off-line systems, where samples are percolated through a sorbent packed in disposable cartridges or enmeshed in an inert matrix of a membrane based extraction disk.

Sorbent types

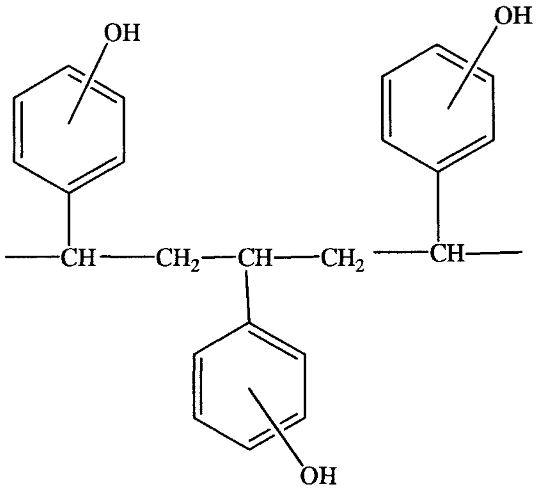
SPE sorbents can be divided into three classes; normal phase, reversed phase and ion exchange. The most common sorbents are based on silica particles, typically 60 μm diameter irregular shaped particles to which functional groups are bonded to alter the retentive properties. Of these the n-alkyl silica e.g. C_2, C_8 and C_{18} are probably the most widely used and are designed to provide a hydrophobic interaction with the analyte. An example of a C_{18} reversed-phase structure is shown in Figure 3.1. However in the manufacture of C_{18}, non-modified silanol groups can be deliberately left to provide secondary polar interactions with the analytes [25]. The problem of extraction of polar analytes is also solved by the introduction of carbon-based sorbents and highly cross-linked styrene-divinyl benzene copolymers. The structure of a hydroxylated styrene divinyl benzene is shown in Figure 3.1.

Normal phase sorbents have polar functional groups e.g. cyano, amino and diol. Ion exchange sorbents have either cationic or anionic functional groups to attract compounds with the opposite charge. For the extraction of nonionic surfactants this discussion will mainly focus on the use of reversed-phase SPE. A more detailed description of this process will follow but first let us consider solvent selection.

Figure 3.1 Typical structures of some SPE sorbents



Typical reversed phase sorbent i.e. C_{18}



Hydroxylated polystyrene divinyl benzene copolymer e.g. Isolute ENV+

Solvent properties

The choice of solvent directly influences the retention of the analyte on the sorbent and its subsequent elution, whereas the solvent polarity determines the solvent strength (or ability to elute the analyte in a smaller volume than a weaker solvent) [26]. The figures for eluent strength in Table 3.1 are taken from Synder [27] and are for adsorption chromatography on silica or normal phase chromatography, where a more polar solvent e.g. methanol has a higher eluent strength. However more applicable to this work is the order of solvent strength for reverse phase chromatography, which is the reverse order of that shown in Table 3.1. It is worth noting that the polarity figures, also quoted from Synder [27], do not increase in the same order as the table is descended.

Table 3.1 Characteristic properties of solvents

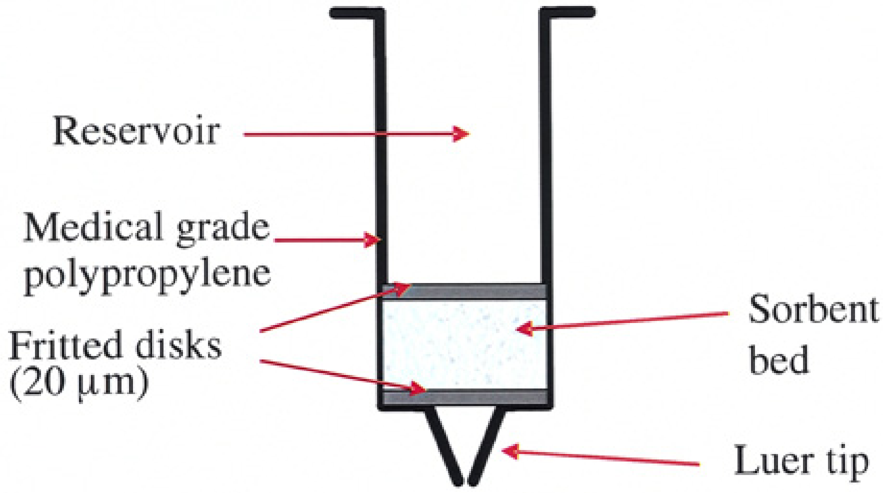
Solvent	Eluent Strength (ε^0)	Polarity (P')	Solvent strength for reverse phase
Hexane	0.00	0.1	Strongest
Dichloromethane (DCM)	0.30	3.1	
Ethyl acetate	0.48	4.4	
Methyl t-butyl ether (MTBE)	0.48	2.5	
Acetonitrile (ACN)	0.52	5.8	
Tetrahydrofuran (THF)	0.53	4.0	
2-propanol	0.60	3.9	
methanol	0.70	5.1	Weakest

The SPE process (reversed phase)

Several devices are used for SPE, cartridge, disk or coated fibre. The most popular configuration is the cartridge or syringe barrel format [27]. This is made from medical-grade polypropylene, chosen for its purity. The outlet normally has a luer tip so that a needle can be affixed to facilitate elution into vials. A diagram showing the main parts of an SPE cartridge is shown in Figure 3.2. The frit to maintain the particle bed is generally made of PTFE or polypropylene with a porosity of 10 to 20 μm to offer little flow resistance. SPE cartridges, being relatively inexpensive, are used a single time and discarded to avoid any risk of contamination.

SPE disks combine the advantages of SPE and membranes. Disks differ in construction from membrane filters and can consist of flexible or expanded PTFE networks filled with silica based packings [27]. Advantages include rapid loading rates (100 mL/min) and absence of channelling. However lower breakthrough volumes can occur compared to SPE cartridges.

Figure 3.2. Design of SPE cartridge

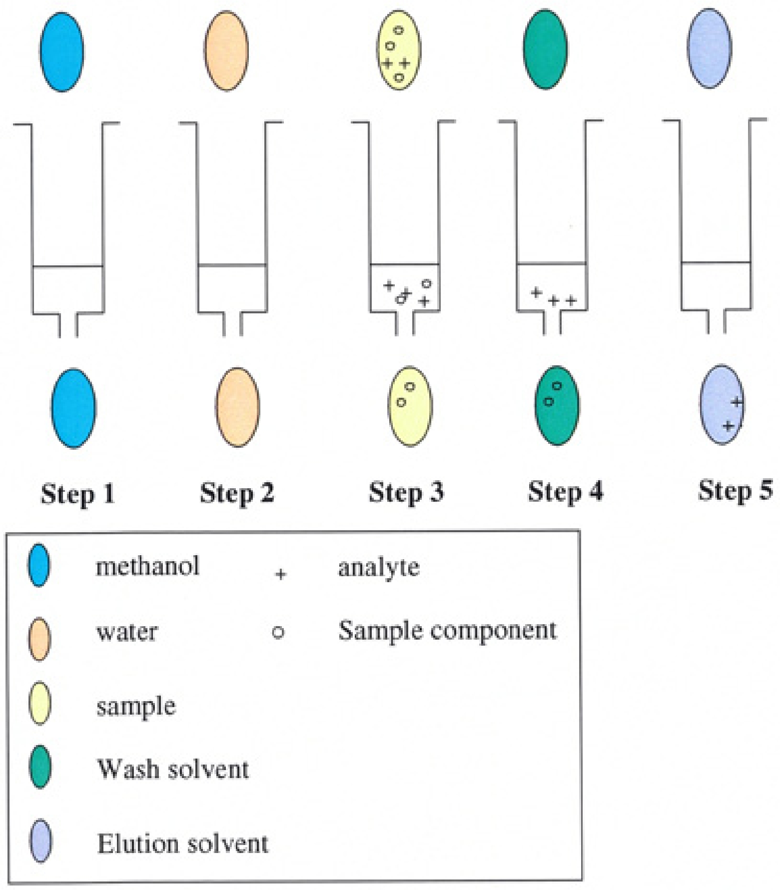


Coated silica fibres are used for solid phase microextraction (SPME). These fibres, coated with phases such as polydimethylsiloxane, are dipped into the solution to be analysed. Mostly these are desorbed in the injector of a GC but can be placed into the injection port of a HPLC where the analytes are displaced by a strong solvent.

The method of operation is the same whether SPE is used in cartridge or disk format. The following description is based on reversed-phase SPE, however similar considerations would be made in normal phase and ion exchange methods. A typical SPE procedure is shown schematically in Figure 3.3. It is considered that the SPE process can be divided into five basic steps described as follows:

Step 1: The sorbent is activated with methanol, typically 1 mL per 100 mg of sorbent [26]. The function of this step serves to remove impurities but also to solvate the hydrophobic surfaces present in reversed phase sorbents.

Figure 3.3 Schematic of the SPE process



Step 2: The sorbent is conditioned with the same volume of de-ionised water.

Step 3: The aqueous sample is applied and the analyte is trapped while the water passes through.

Step 4: A clean-up step can be added to remove co-extracted interferences, typically water with a small amount of organic solvent is used, i.e. weak enough not to elute the analyte.

Step 5: This step involves drying of the sorbent, typically by drawing air through by vacuum suction, and elution with organic solvent. A volume of 0.5 to 1 mL is typically required per 100 mg of sorbent [26].

In the dry state the alkyl chains are twisted and collapsed on the surface and on contact with a suitable solvent they spread open to form a bristle [28]. This ensures good contact between the analyte and the bonded phase in the adsorption step. Interactions between the analyte can be considered as hydrophobic or van der Waals binding forces between apolar groups that are enhanced in aqueous medium

[28]. Owing to the high affinity among water molecules (hydrogen bonding), apolar molecules get excluded and the carbon skeleton of the analyte binds with the carbon skeleton of the bonded phase. These weak binding forces can be easily broken by replacing water molecules surrounding the analyte with less polar molecules, which is the principle of the elution step.

Automation

The SPE process can be performed sequentially for up to 24 cartridges at a time using vacuum extraction units. The whole sequence can also be fully automated off-line using commercially available systems such as the ASPEC from Gilson [29] and AutoTrace and RapidTrace from Zymark [30].

Figure 3.4 Multi-cartridge vacuum manifold system

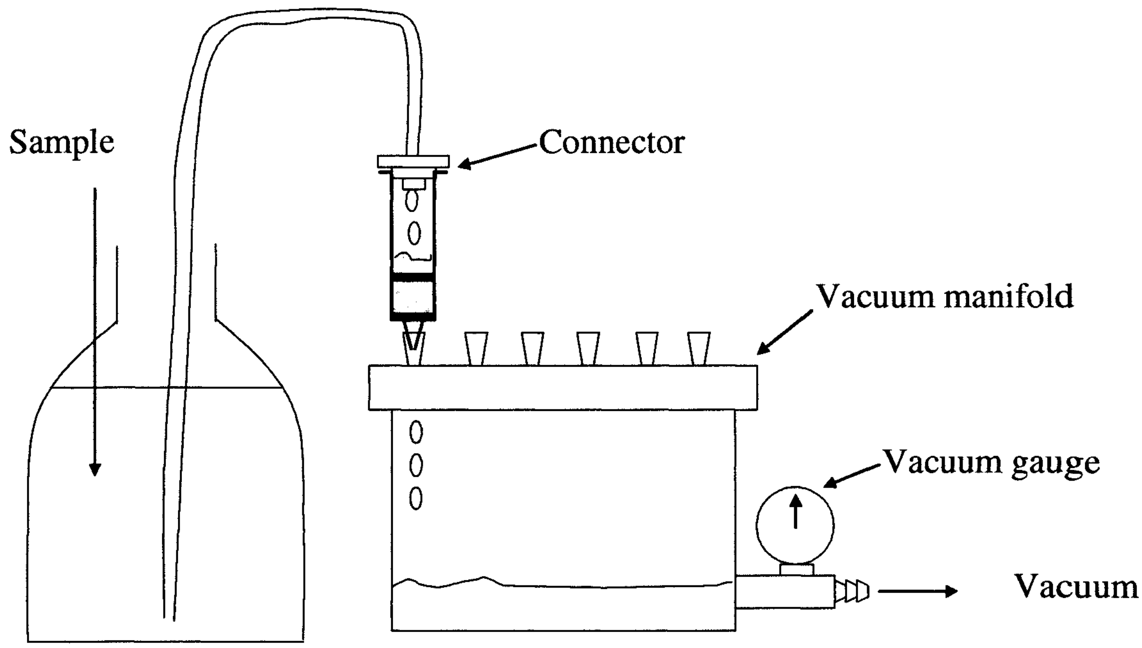


Figure 3.4 demonstrates a typical set up of a vacuum manifold, which loads samples using negative pressure. Advantages of automated systems is that exact flow rates for conditioning, load, rinse and elute can be set by the user because a controlled positive pressure is used to push samples and reagents through the SPE columns. This in turn can lead to better reproducibility of the technique with fewer outliers occurring [28]. Due to volumes of samples handled the automation has been introduced differently for biological and environmental matrices. A typical volume

for a biological sample is 1 to 10 mL, whereas for environmental samples 100-200 mL is more typical [25]. The ASPEC can couple on-line SPE with LC so that the whole analysis is automated. The AutoTrace however will process six samples off-line but is designed to load larger sample volumes, of up to 1 litre, onto the SPE cartridge.

3.2.3 METHODOLOGY AS APPLIED TO AEs

Dubey et al. [8] analysed nonionic surfactants in effluents and activated sludges by pretreatment with a foam sublation or gas-stripping technique. This provided a gross separation with polyethylene glycols eliminated in the water layer and other surfactants passing into an ethyl acetate layer. An ion exchange step was then used to eliminate ionics with the remaining nonionics analysed by HPLC. This method was quoted to give good recoveries with the ethoxylate distribution of an extracted sample reflecting that of a standard. The gas stripping method uses a stream of nitrogen to transport surfactants from the aqueous phase to the organic phase but the equilibrium between the two phases is governed by the same factors as in liquid-liquid extraction [31]. It is reported that this methodology is efficient in processing large volume water samples but long chain EOs are lost in both gas-stripping and liquid-liquid extraction with ethyl acetate [31].

Two pieces of work worthy of note are those by Evans et al. on the analysis of AEs in environmental samples by thermospray LC/MS [11] and ESI LC/MS [12]. In these two methods speciated EO recoveries for EO_{2-18} are quoted for C_{12-15} alkyl chains. These methods utilised SPE on a 1 g C_8 SPE cartridge for the analysis of influents and effluents. Final elution of the cartridge was carried out with 8mL methanol followed by 4 mL of isopropanol. Mean recovery of AEs at 10 μg/L in final effluent was good but there was some variability in the individual EO recovery (45 – 194 %).

In a more recent evaluation of SPE procedures, for the analysis of AEs and alkylamine ethoxylates used as adjuvants in pesticide formulations from ground water and surface water, Krogh et al. [16] used a Porapak RDX cartridge, eluting with 5 mL methanol: ACN (1:2) and 5 mL methanol: DCM (1:4). Recoveries of

AEs at 16 – 60 ng/L ranged from 35 – 93 %. Porapak RDX is a divinylbenzene-vinylpyrrolidone phase. Others cartridges were evaluated, including two types of C_{18}, Oasis HLB and Isolute ENV. The Porapak and Oasis HLB initially gave the best results for the two types of nonionic surfactant analysed but blocking of the Oasis cartridge during the loading process meant the former was preferred. It was reported that poor recovery of analytes with longer alkyl chains (i.e. higher log K_{ow} values where K_{ow} is the octanol-water partition coefficient) was obtained. Recoveries were optimised by the application of a polar elution solvent followed by a more apolar elution solvent.

In an interesting piece of work by Cretier et al. [32] good recoveries of AEs were obtained from raw waste water using isolation on a styrenedivinylbenzene disk. They also employed an extraction cell to improve recovery of analytes by eluting the dried disk with methanol at 100 °C. Analytes ranging from 10 to 16 alkyl carbons and 1 to 25 ethoxylate groups were recovered at more than 70 % with a limit of quantification between 0.05 and 4 μg/L. The wastewater, a sewage influent, which was immediately preserved with 37 % formaldehyde (7.5%), was analysed in 20 mL aliquots some of which had been spiked and aged for 75 days before analysis. The 20 mL volume was the maximum amount of this type of sample that could be loaded without plugging of the disk.

A novel approach to the analysis of AEs utilised SPME with LC and fluorescence detection. This work was carried out by Aranda and Burk [33] and utilised an online derivatisation using 1-naphthoyl chloride (1-NC). The SPME fibre was stirred in AE solutions for 1 hr before air drying in the headspace for 5 min. The fibre was then transferred to the SPME interface where it was reacted with 1-NC and a catalyst in a pyridine solution. The derivative was then injected onto the LC, where AEs were determined with a detection limit of 0.1 mg/L. However problems were reported which included the reaction of the polydimethylsiloxane-divinyl benzene fibre with 1-NC and also that the adsorption of the analyte onto the fibre was influenced by the presence of other compounds. This would be an important consideration if this technique was used in the analysis of environmental samples.

Dunphy et al. [19], using the pyridinium derivatives described earlier, made a very thorough investigation into the sample preparation procedures for AEs in sewage influent and effluent. The method was designed to analyse all species including the alcohol and low ethoxylates, so it was also necessary to optimise the extraction of the species in the SPE process. The removal of all water from the final extract was essential to avoid quenching of the derivatisation reagent. They concluded that some of the more volatile AEs ($C_{12\text{-}15}EO_{0\text{-}3}$) were significantly lost on drying after SPE so they eluted the AEs in two fractions to overcome this problem. Low ethoxymers were eluted with ACN with this fraction being set aside and not taken to dryness. A second elution was then carried out with 1:1 v/v methanol/ethyl acetate containing 2 % water, which was dried to remove the solvent including residual water. The two fractions ACN and dried residue were then combined and derivatised. The pyridinium reagent was thus not consumed by reaction with protic solvent. However there were low recoveries particularly evident in the C_{18} AEs (24–65 %).

Other workers have successfully used graphitised carbon black (GCB) cartridges for the analysis of AEs and their biotransformation products [34] and also the simultaneous determination of AEs and alkyl phenol ethoxylates (APEs) [35]. AEs were typically eluted using mixtures of DCM/methanol (70/30 v/v) [35] and (80/20, v/v) [34]. For the biotransformation study, acidic metabolites were eluted with DCM/methanol (80/20, v/v) acidified with formic acid, 50 mmol/L. The GCB cartridge is often presented as a more retentive reversed phase sorbent than C_{18} silica but with a very different retention mechanism [25]. Compounds are retained by both hydrophobic and electronic interactions so that both non-polar and very polar analytes can be retained from water. The structure of GCB is of crystalline graphitic sheets held together by weak Van der Waals forces. However positively charged active centres on the surface enable the GCB to behave as an anion exchanger [36].

The fact that many different approaches have been tried to extract AEs from environmental samples indicates the difficulty in obtaining a procedure to reflect all the individual compounds present in the samples. Approximately one thousand different compounds may be present in a mixture of nonionic samples in the aquatic environment [31].

3.3 Sample preparation for solid samples

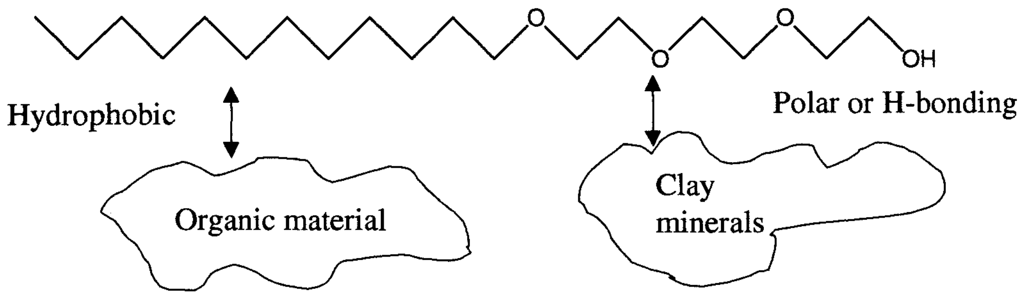
3.3.1 GENERAL METHODOLOGY

Surfactants are discharged into the sewage system where removal is either by biodegradation or adsorption onto sewage sludge. For risk assessment purposes, the eventual discharge of the sludge must be considered. This may be in effluents to a receiving river, (such that it ends up in sediment), or it may be used on land as a fertiliser. Hence screening methods for the analysis of surfactants in sludge, agricultural soil and sediment are required. Surfactants adsorbed onto river sediments were found to biodegrade rapidly and at least at the same rate as when they were dissolved in river water [37]. This finding was made in monitoring studies for linear alkyl benzene sulphonate (LAS). In fact most of the data available for river sediments is for LAS or alkyl phenol ethoxylates (APEs) [38]. Cavalli et al. [34] claimed that their data on AEs in river sediments was the first of the type.

A review by H. Klotz [39] highlights some important considerations in sediment analysis. For AEs typically 8 % v/v formalin is recommended to preserve the sample. If sludges and sediments are dried by evaporation, some surfactants, for example AEs and APEs with low degrees of ethoxylation may be lost. A better approach for drying may be centrifugation followed by mixing with sodium sulphate. Sediments should also be classified by sieving into the following fractions: clay (<2 μm), fine (<20 μm) and coarse (20-60 μm). Analysis from these fractions is all that is necessary as sand >60 μm contains very little organic contamination.

The interaction of AEs with the sediment is dominated by a hydrophobic mechanism. Kiewiet et al. [40] studied sediment/water partition coefficients for eleven different AEs, C_xEO_y where x represents the number of carbon atoms in the alkyl chain and y the number of ethoxylate groups. As x increased so did the sorption to sediment but somewhat more surprisingly an increase in y also yielded an increase in sorption. Another review by Krogh et al. [4] explains interactions that are possible with the hydrophobic alkyl chain absorbing to organic matter, whereas the hydrophilic ethoxy chain might bind via hydrogen bonding to the more polar clay minerals (see Figure 3.5).

Figure 3.5 Possible interaction of AE with sediment

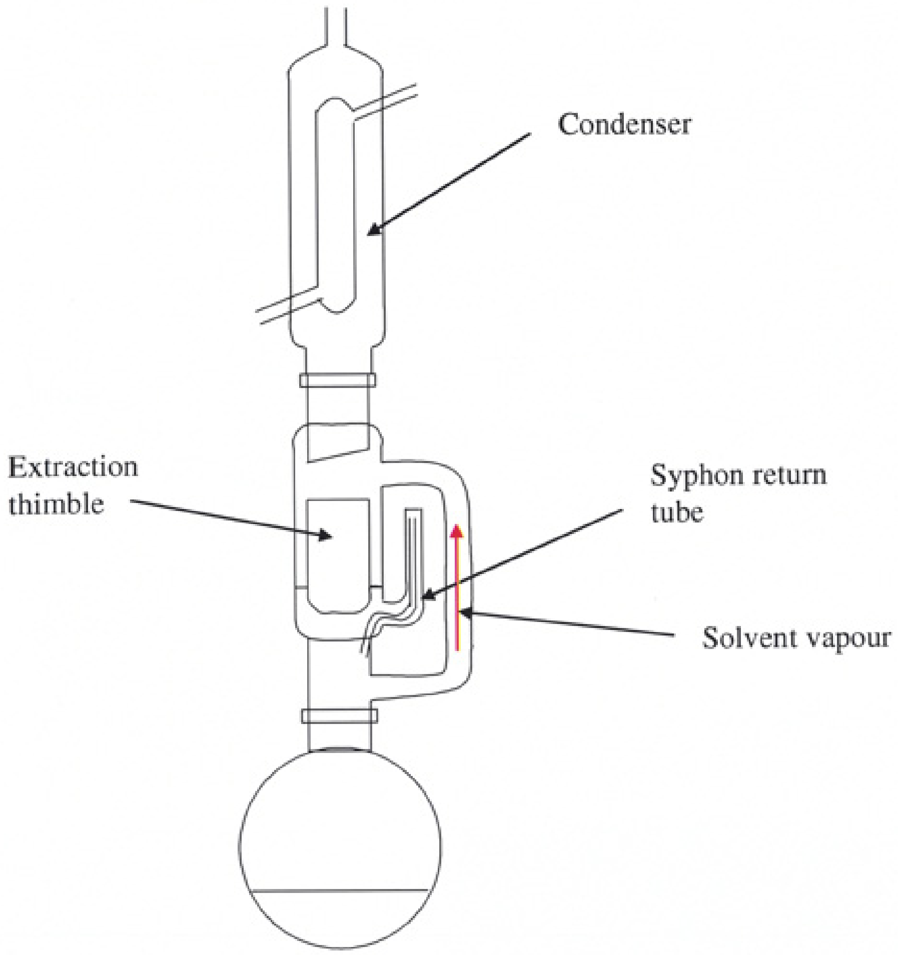


The interactions described will need to be considered when extracting AEs adsorbed on a sediment or sludge into a suitable solvent. Liquid-solid extraction for the removal of analytes can be divided into two main groups: Soxhlet, where heat is required and others where extraction takes place by some form of agitation i.e. shaking or ultrasonic. Soxhlet extraction is a very old established method dating back to the mid-nineteenth century when it took its name from Baron von Soxhlet [26].

Soxhlet apparatus can vary in design from those where the hot vapour passes over the sample to designs where the hot solvent by-passes the sample and condenses, allowing cooled solvent to drip back through the solid in the extraction process. The apparatus is shown in Figure 3.6, but nowadays it is common to use an automated Soxhlet extraction (commercially known as a Soxtec) which has been designed in an attempt to speed up the process. Soxtec apparatus allows the extraction thimble to be immersed in boiling solvent for a period of time before raising the thimble to allow the more conventional extraction with solvent condensing back through the sample. Finally the solvent can be evaporated off within the apparatus, enabling sample concentration.

Ultrasonic extraction uses sound waves to agitate the sample immersed in organic solvent. Typically a sonic bath is used which enables several samples in beakers containing the required amount of solvent to be extracted simultaneously. Repetition of the procedure and combination of filtered or centrifuged extracts is normally required in this procedure. The ultrasonic bath will normally be temperature controlled but the sonication process will typically generate some heat as well.

Figure 3.6 Soxhlet apparatus



More modern techniques include accelerated solvent extraction (ASE) which attempts to automate, speed up and reduce solvent usage when extracting solid samples. This will be discussed in more detail in the next section.

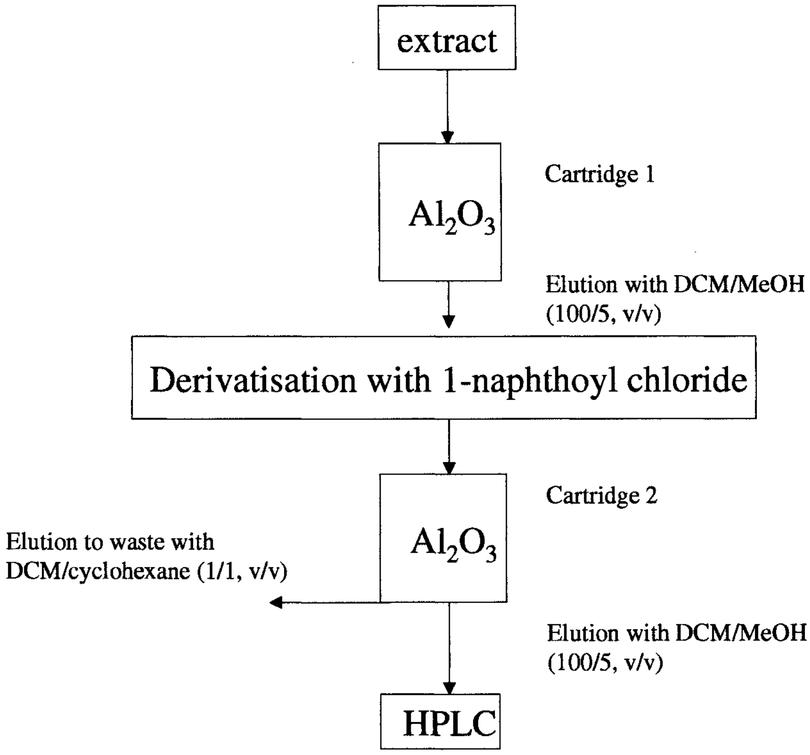
3.3.2 APPLICATION OF THE SOLIDS METHODOLOGY TO AEs

Krogh et al. [16] this time looking at AEs in agricultural soil developed a method using pressurised liquid extraction (PLE), synonymous with ASE. The most important parameters to be optimised in this technique are temperature, polarity of the solvent, extraction time and water content of the matrix. The choice of solvent is essential to improve the effectiveness of the method, with analyte retention also dependent on the type of soil. Pressure is of minor importance for a dry soil but elevated pressure may be required to maintain the extraction efficiency with higher moisture content soils. AEs were reported to be extracted using methanol (150°C and 1500 psi), although the most apolar AE determined was $C_{18}EO_6$. An SPE procedure was evaluated as a clean-up step but did not result in any overall improvement of the analytical method. AEs were recovered at 47-106 % with detection limits in the range of 7-13 μg/kg.

Chiron et al. [9] analysed sludge samples, available for use in agricultural fields (fractions below 150 μm) using Soxtec extraction. After oven drying at 40°C, 10 g samples were extracted with 50 mL of methanol, immersed during 45 min and then rinsed for 4 hr. The methanolic extract was then diluted in water in the ratio water/methanol 70/30 v/v and passed through a C_{18} 1g 6 mL cartridge before final analysis by LC/MS. Total C_{12}, C_{13}, C_{14} and C_{15} AEs were quantified at 2.8, 4.1, 5.4 and 8.5 mg/kg respectively in sludge of this type.

In a survey on sediments in the River Po in Italy by Cavalli et al. [38], samples were freeze-dried before extracting a 5 g sample with methanol (50 mL) by sonication for 30 min at 40 to 50°C. This extraction was repeated twice, combining centrifuged extracts and concentrating to a volume of 25 mL. This extract was then subjected to a clean-up procedure on an alumina cartridge, followed by derivatisation and further clean-up (Figure 3.7). AEs were determined in the range 0.1-1.0 mg/kg and were considered an overestimate due to the methodology used which summed all signals appearing in the AE chromatographic window of the LC-fluorescence method. The cleanup procedure has been reported to suffer a loss of AEs with less than 3 EO units [39].

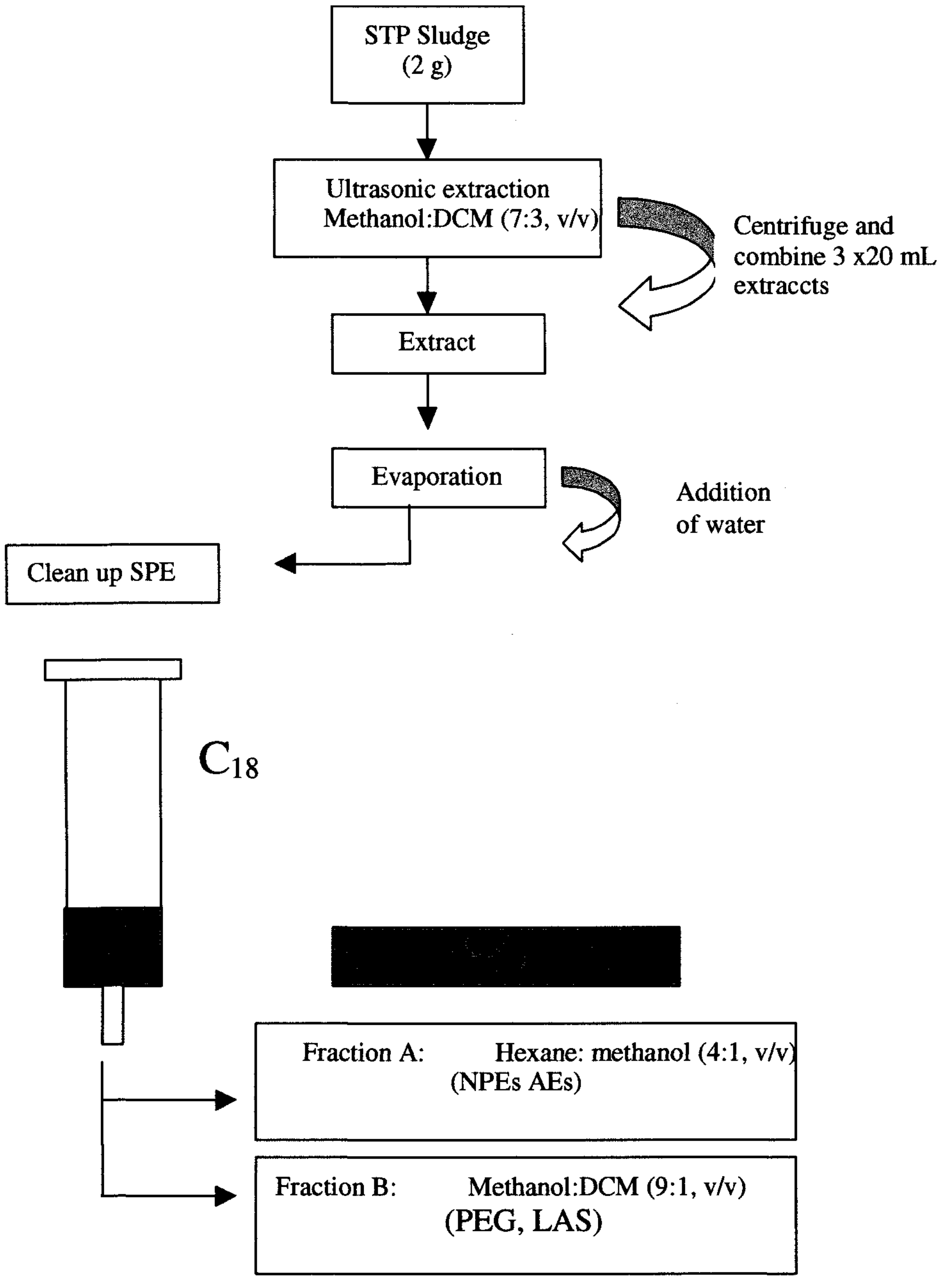
Figure 3.7 AE methodology using alumina clean up and LC fluorescence.
Adapted from Klotz [39]



The methodology represented in Figure 3.7 has been applied to AEs in sediments [38]. In a recently published paper, the method was used in a collaborative study for the analysis of sewage sludge [41]. In work by Tolls et al. [42] the same clean up was applied to extracts of AEs from fish samples. More interestingly however they used matrix solid phase dispersion (MSPD) to isolate the AEs from the fish tissue. This involved grinding together, using a mortar and pestle, 4 g of octadecylsilica (ODS) per 1 g of homogenised fish to eventually form a dry powder. By transferring to a suitable empty column and sequential elution with solvents, whereby hexane elution was first of all discarded the AE were eluted in ethyl acetate and ethyl acetate: MeOH (1:1, v/v) fractions. These fractions were then combined, dried, resuspended and taken through alumina clean up procedures already described. In this work recoveries of AE greater than 75 % are described with extracts sufficiently purified to detect AE using LC fluorescence without interferences compromising quantitation.

Petrovic and Barcelo [43] studied AEs in sewage sludge along with other groups of compounds including anionics, nonylphenol ethoxylates (NPEs) and their degradation products. The overall scheme is represented in Figure 3.8. Final analysis was performed by LC/APCI-MS and AEs were found in the sludge at levels of 10-190 mg/kg.

Figure 3.8 Scheme for extraction of AEs from sewage sludge using ultrasonic extraction



3.4 Conclusion

This chapter describes past and current methodology for the extraction and analysis of AEs and focuses on instrumental techniques as well as extraction from aqueous and solid samples. In Chapter 7 the development and optimisation of an SPE method suitable for extracting AEs from sewage effluent and influent samples is described. No methodology examining detailed recovery of AEs ranging from 12 to 18 alkyl carbons and 0 to 20 ethoxylate groups is present in the literature for solid samples. An investigation into the extraction of AEs, from sediment and sludge with subsequent derivatisation with phthalic anhydride and analysis by LC/MS is presented in Chapter 8.

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Chapter 4

INTRODUCTION: LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

4.1 LC/MS the hyphenated technique

4.1.1 HISTORY OF LC/MS

The technology behind the hyphenated practice of liquid chromatography/mass spectrometry (LC/MS) can be traced back to work performed in the 1960s and through the mid – 1980s. However the true commercialisation and acceptance of the technique was probably in the 10 years that followed this. In tracing the development to today's practice of the successful application of LC/MS it is important to highlight some critical steps in its creation [1].

- The atmospheric pressure interfaces derived from Dole's work with electrospray in 1968 [2].
- Horning's work in 1974 with solvent modified electrospray or atmospheric pressure chemical ionisation [3].
- The development of thermospray from work published by Blakely and Vestal in 1983 [4].
- Willoughby and Browner's work with particle beam interfaces in 1984 [5].

The technique has been developed more by the efforts of the MS community rather than chromatography practitioners. The membership of the American Society for Mass Spectrometry (ASMS) doubled from 1987 to 1996, with the emergence of LC/MS as an analytical tool largely responsible [1]. The developments also brought the technique into the hands of bench-top users as opposed to traditional mass spectrometrists.

The motivation behind the attention paid to the development of LC/MS was brought about by the success of commercial gas chromatography/mass spectrometry (GC/MS) systems and the necessity to extend the analyte range to more polar and thermally labile molecules. Experimentally the major problem experienced in the early development of LC interfaces was the fact that a typical LC flow of 1 mL/min would generate 1-4 L of gas (at 1 atm) when introduced into a MS where the vacuum was 10^{-6} torr. This translated to approximately 100 times the gas flow experienced by early workers coupling GC to MS [6]. Early attempts to deal with this included moving belt interface (MBI), direct liquid introduction (DLI) and thermospray (TSP).

4.1.2 MOVING BELT INTERFACE (MBI)

Scott et al. [7] designed a system using a moving wire to introduce solvent and solute into the MS source via vacuum locks where the vaporisation of the solvent was accomplished. This was followed by vaporisation of the solute by passing a current through the wire. The sample transfer efficiency of this system was only about 1 %. Modifications and improvements were made to the system by McFadden [6] which used a polyimide Kapton belt allowing direct deposition of LC eluent, differentially pumped vacuum locks and infra red heaters to remove solvent, followed by flash vaporisation of the solute as it traversed the entrance to the MS.

4.1.3 DIRECT LIQUID INTRODUCTION (DLI)

This simple design was pioneered by Tal'Rose et al. [8] and Baldwin and McLafferty [9]. The interface was compatible with direct probe GC/MS systems. In common with devices of this type is the 3 to 5 μm opening in the probe tip to the MS source, allowing 5 to 15 μL/min to enter the source controlled by a volume restrictor based on pressure across the orifice. The substitution of supercritical fluid chromatography for LC enabled introduction of a bigger proportion of the mobile phase as the carbon dioxide used decompressed to a gas and was more readily pumped away [6].

4.1.4 THERMOSPRAY (TSP)

The LC/MS interfaces described previously can at best be looked at as partial solutions with the two main criteria i.e. handling large flows of highly polar solvents and the ability to handle thermally labile compounds or compounds with low volatility remaining unsolved. Thermospray (TSP) mass spectrometry developed by Vestal [4] provided an immediate solution to both these problems. The flow from the LC is directed into a heated hypodermic tube, which acts as a vaporiser. The solvent, which usually contains buffers such as ammonium acetate, exits the tip of the vaporiser in a fine spray. Ions are formed from this electrolyte as the solvent evaporates which can then react with the solute. Typically TSP exhibits ions of the form $[M+H]^+$ and $[M+NH_4]^+$. This process thus represents a new method of ionisation. TSP removed the ionisation from the high vacuum (10^{-6} torr) to rough vacuum levels (10^{-2} torr).

4.1.5 PARTICLE BEAM (PB)

The MBI was for some time the only means of acquiring classical electron impact (EI) spectra from LC eluates, but was mechanically complex, prone to memory effects and had a limited sample volatility range [10]. The introduction of the particle beam (PB) provided another alternative. The transfer of neutral analytes from column to MS is accomplished by aerodynamic means with the interface providing aerosol formation, desolvation and momentum separation. The technique however has problems of poor sensitivity, band broadening and non-linearity.

All of the techniques mentioned so far are deficient in one or more of the aspects of performance demanded by LC users – sensitivity, applicability, stability and ease of use.

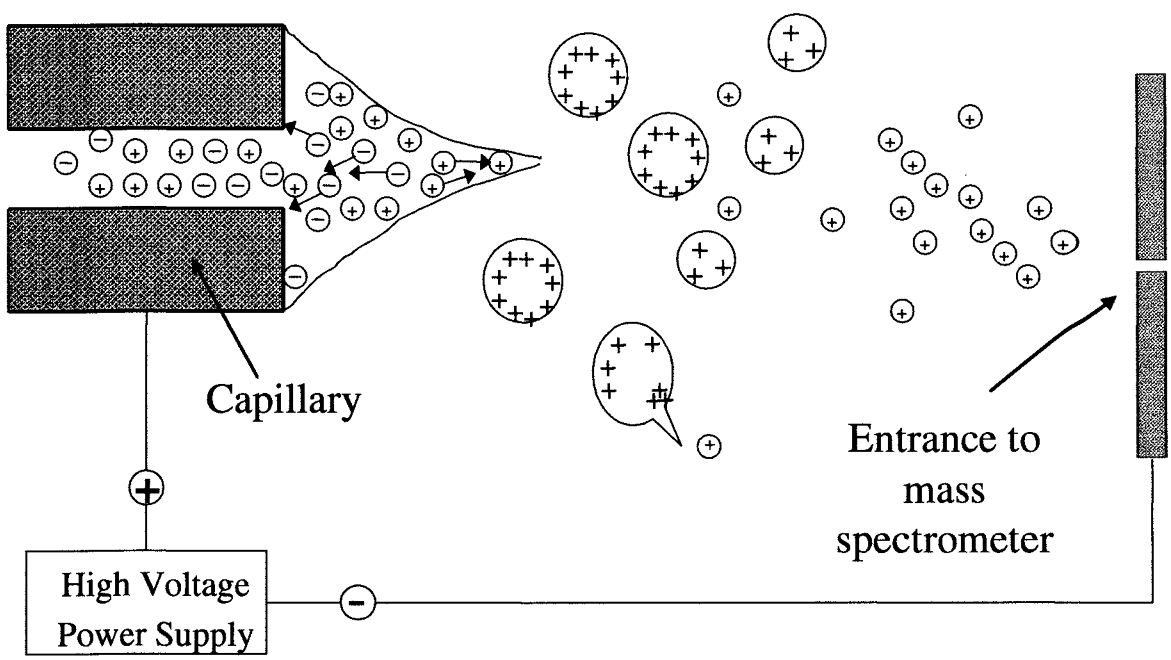
4.1.6 ELECTROSPRAY IONISATION

The impact of electrospray ionisation (ESI) on modern mass spectrometry is immense and enabled the design of a more stable and universal LC/MS interface. A foreward to Cole's book on Electrospray ionisation mass spectrometry [11] written by Fenn gives a very interesting account of his involvement with the early days of ESI and pays particular reference to Dole in his work on spraying solutions of macromolecules in 1968 [2,12]. Unfortunately Dole did not live to see the full fruition of his work. From the initial work by Dole, several research groups, most notably Fenn [13] developed applications of ESI/MS for the analysis of biomolecules. Multiply charged ions of proteins and peptides can be produced thereby rendering them amenable to mass analysis using a quadrupole or ion trap with a mass-to-charge (m/z) range of around 2000. The other key feature of ESI is that the ionisation is carried out at atmospheric pressure, termed atmospheric pressure ionisation (API). The process is compatible with most chromatographic separations, mobile phases and volatile buffers. In ESI the key processes involved in the API interface are the transformation of ions in solution to ions in the gas phase. HPLC solvent must be removed before the gas phase ions can enter the high vacuum of the MS (10^{-5} torr). Once inside the MS the ions are detected on the basis of their m/z ratio.

In more detail the ESI process normally begins with charged polar analytes in the LC eluent. This passes through a fine capillary and the combination of an electric field and high gas flow rates causes the formation of an aerosol of charged droplets. Examined in more detail [11], the presence of an electric field applied to the tip of a capillary containing electrolyte ions leads to the formation of a dipolar layer at the meniscus of the liquid. When the capillary is positive, enrichment of positive ions in the double layer close to the surface have a destabilising effect on the meniscus, which causes a cone and liquid jet which emits positively charged small droplets. The charged droplet stream leads to an electrical current generally in the region of 1 μA. This process is more clearly demonstrated in Figure 4.1. Evaporation causes the droplet to decrease in size. At the critical point, the Rayleigh limit, the repulsive forces on the charged surface of the drop exceed the surface tension and cause the droplet to explode into smaller charged drops.

There are two theories for the formation of gas-phase ions from the very small charged droplets [11]. The first mechanism proposed by Dole [2] and later supported by Röllgen [14] suggests that small droplets containing only one ion will eventually lead to a gas-phase ion on subsequent evaporation of solvent from the droplet. This model is referred to as the *charged residue model.* Iribarne and Thomson [15], predicted that after the radii of droplets reach a critical size direct ejection of ions from the droplet become possible in a model called *ion evaporation.* This process becomes dominant over coulomb fission for droplets with radii ≤ 10 nm.

Figure 4.1 Schematic diagram of the major processes occurring in electrospray ionization.
Adapted from Kebarle et al. [16]



Two different source designs are in common use to transport ions formed at the atmospheric pressure region into the first stage of the low-pressure region [17]. In one design a heated glass capillary is used as an orifice, in another ions are sampled through a small orifice skimmer. The ions pass through further low pressure regions separated by skimmers before entering the high vacuum mass spectrometer.

Both positive and negative ions can be analysed depending on the capillary voltage selected.

ESI is a very mild process leading to the formation of intact molecular species with almost no fragmentation. The technique is capable of analysing low to high mass compounds. For the high mass compounds a series of multiple charged ions is formed with resultant m/z values brought into the mass range of the ordinary mass spectrometer. Structural information can be obtained by applying voltages to the skimmer or heated capillary of the systems described.

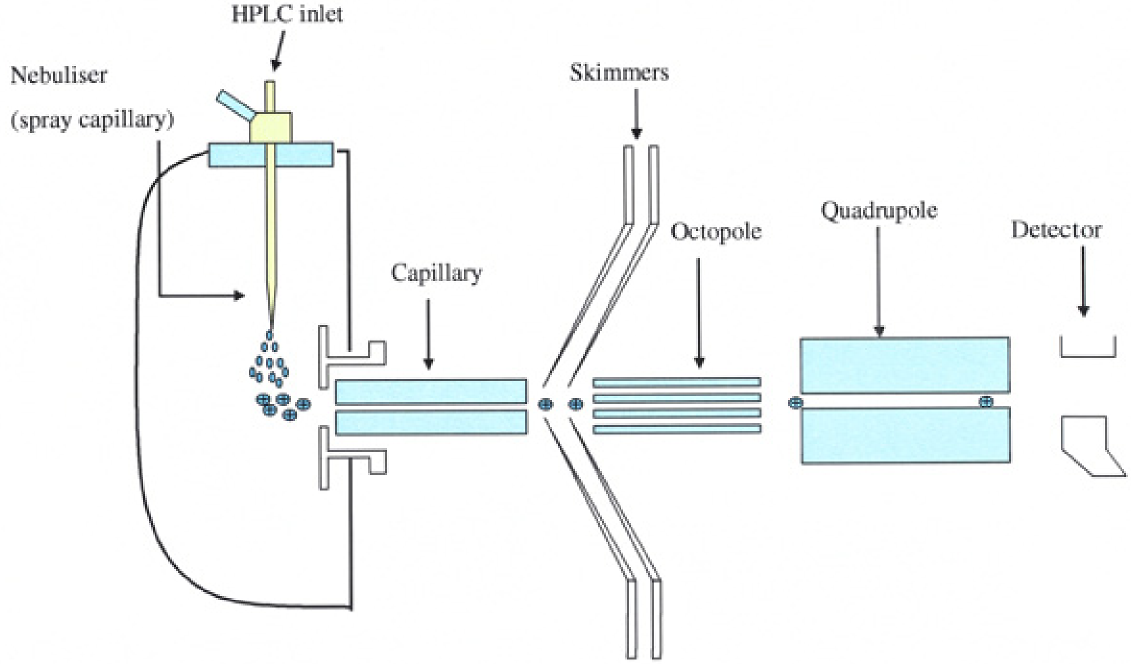
The composition and flow rate must be controlled for effective electrospray. A flow rate of 1 – 10 μL/min is required for stable spray formation. A fluid with high surface tension such as water is difficult to electrospray. However many polar solvents such as methanol, acetonitrile, ethanol and isopropanol are suitable for electrospray operation. In contrast normal phase solvents which are non-polar in nature e.g. hexane are not easily dispersed in the electrospray process and difficult to use. High concentrations of salts and ion pairing reagents can also reduce the signal obtained. Generally a decrease in sensitivity is obtained for an increase in electrolyte concentration.

Several designs of the ESI interface have evolved to circumvent the problems associated with the traditional source. Pneumatically assisted electrospray, also termed as ion spray, uses a flow of nebulising gas to help shear droplets, allowing increased flows of 200 to 400 μL/min to be sprayed [14]. In addition, sources contain heated regions and auxillary gas flows to assist in the evaporation process. Another approach for decreasing the number of desolvated droplets was the introduction of an orthogonal ESI source [18]. Up until then electrospray systems utilised an in-line geometry where both charged droplets and ions were directed into the optical axis and the detector. In the new design the spray geometry was changed to be orthogonal to the optical axis, thereby only allowing ions and not charged droplets to enter the vacuum system. A schematic diagram of this ESI interface is presented in Figure 4.2.

4.1.7 ATMOSPHERIC PRESSURE CHEMICAL IONISATION (APCI)

The exploration of APCI for LC/MS started in the early 1970's with the work of Horning et al. [3]. In 1974 APCI was developed using either a ^{63}Ni foil or corona discharge needle as the source of electrons. A good review of the development of APCI is presented by

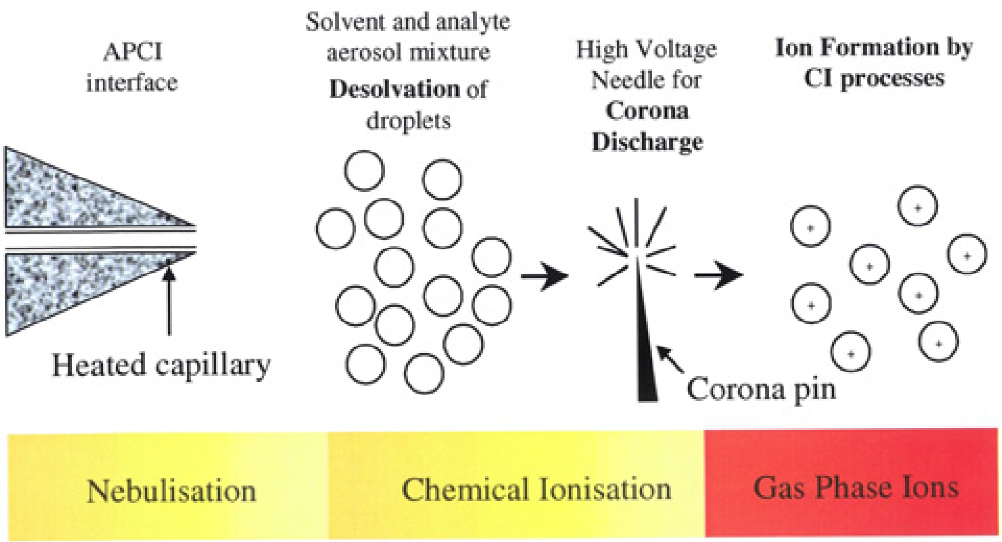
Figure 4.2 Schematic diagram of an orthogonal electrospray system.
Adapted from Imatani et al. [19]



Niessen [20]. The commercialisation of APCI follows on from the advent of good API technology and pneumatically assisted electrospray technology. Instruments are nowadays generally equipped with both electrospray and APCI interfaces.

By thermally evaporating the solvent and including a high voltage discharge needle, it is possible to ionise low polarity analytes, a technique referred to as atmospheric pressure chemical ionisation. In APCI the eluent from the LC is sprayed through a heated capillary, typically at 250 to 450 °C. The heat vaporises the liquid and the resulting gas phase solvent molecules are ionised by electrons discharged from a corona needle. The charge on the solvent is then transferred to the analyte in subsequent chemical reactions (chemical ionisation). Typical schematic diagrams showing the APCI process and typical instrumentation are included in Figure 4.3 and 4.4, respectively. Ions, once generated, are transported to the MS as described for ESI.

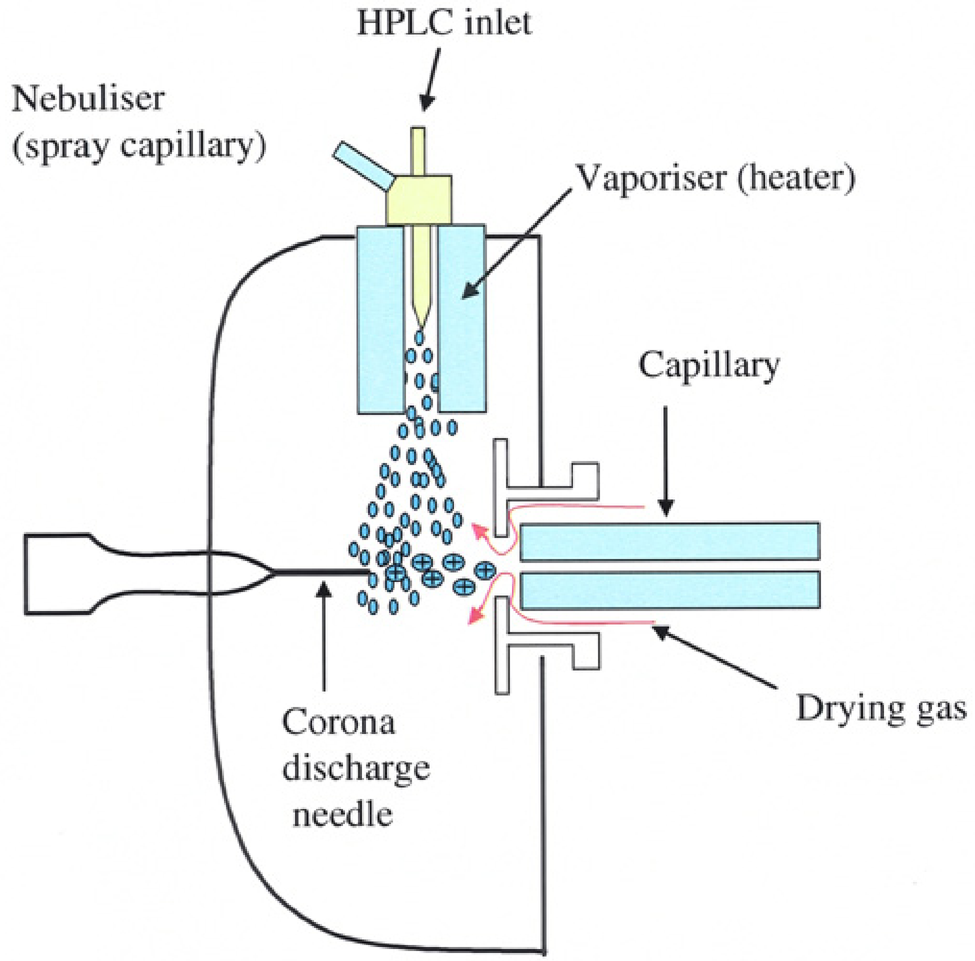
Figure 4.3 Schematic diagram of the APCI process.



4.1.8 *ATMOSPHERIC PRESSURE PHOTOIONISATION (APPI)*

Atmospheric pressure photoionisation (APPI) has been introduced recently by Bruins et al. [21]. The vaporisation process is identical to APCI but ionisation is brought about by using a discharge lamp to generate photons in a narrow range of ionisation energies. Analyte molecules are selectively ionised by these photons in preference to solvent molecules. Typical solvents have high first ionisation potentials [21]. The addition of a dopant, an additive to the mobile phase which is itself first ionised, leading to subsequent ionisation of analytes has also shown good results [21]. Toluene and acetone are examples of dopants, which can be added post-column. The applicability of this technique has shown promise for non-polar compounds and lower flow rates (<100 μL/min), where APCI sensitivity is reduced [18]. As a relatively new technique there are relatively few reports on APPI and its application to real samples [22]. APPI applicability has been shown to overlap with GC/MS e.g. polyaromatic hydrocarbons (PAHs) and the technique certainly appears to be more useful for non-polar molecules [22].

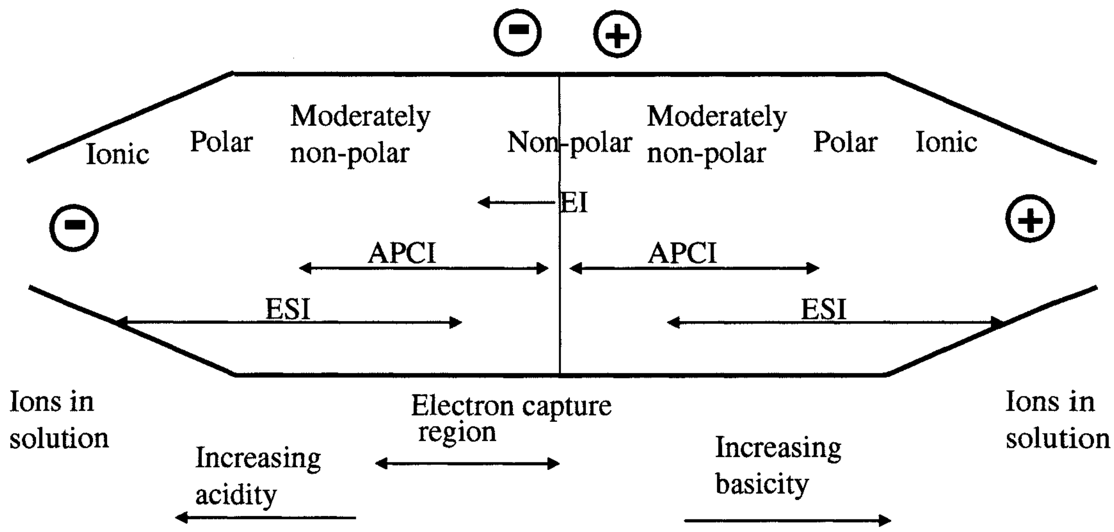
Figure 4.4 Typical APCI interface



4.1.9 SUITABILITY OF LC/MS TECHNIQUE

As discussed previously most API mass spectrometers offer two interfaces ESI and APCI, both of which can be operated in the positive and negative ion mode for protonated and deprotonated ions, respectively. Acid base reactions are the most frequently observed ionisation mechanisms for strongly polar analytes [22]. The addition of alkali ions such as Na^+ or other ions such as NH_4^+ in the positive ion mode or Cl^-, formate or acetate in the negative ion mode can also be used to enhance the ionisation of polar compounds [22]. For a given analyte an appropriate selection can be made on the basis that ESI transfers ions from solution into the gas phase, whereas APCI is more suited to non-ionic analytes. Thurman and co-workers developed a so-called ionisation continuum diagram based on the analysis of pesticides [23] (see Figure 4.5).

Figure 4.5 Ionisation continuum diagram. Adapted from [23]



4.2 Mass spectrometry

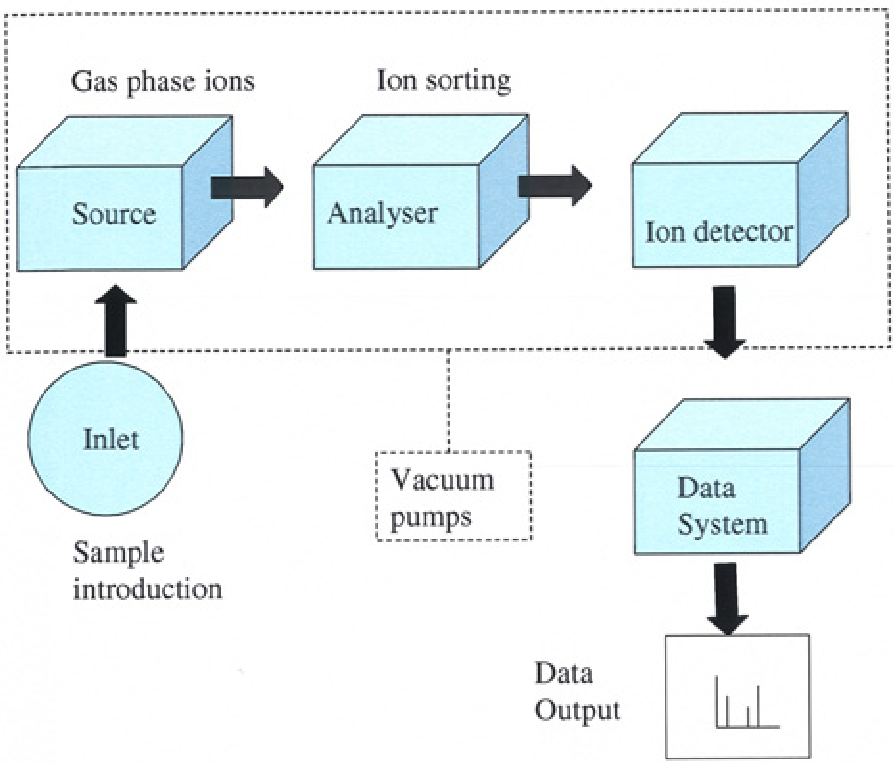
4.2.1 INTRODUCTION

A brief overview of the technique will be provided with particular reference to those aspects necessary for the application of LC/MS. In terms of a mass spectrometer, being a detector for LC, let us first consider some of the advantages provided. A conventional detector, such as an ultra violet (UV) detector, although providing some characteristic data does not provide unequivocal identification of a compound. If we consider chromatography to be the separation of components in a mixture to allow identification and quantification of some or all of them, then by using a UV detector there is the possibility that one or more analytes may have the same retention characteristics. The advantage of a mass spectrometer is clear in that it can provide absolute identification, including molecular weight and structural information, or at least dramatically reduce the number of possible structures for the analyte.

4.2.2 *WHAT IS A MASS SPECTROMETER?*

A mass spectrometer is an instrument that measures the mass-to-charge ratio (*m/z*) of individual molecules that have been converted to ions, i.e., molecules that have been electrically charged [24]. The masses involved are so small that a convenient unit is required for the mass of individual molecules (mass of a single hydrogen atom is approximately 1.66 x 10^{-24} g). This unit is referred to by biochemists as the Dalton (Da), where 1 Da = 1/12 of the mass of a single atom of the isotope carbon-12 (^{12}C). This follows the accepted convention of defining the ^{12}C isotope as having exactly 12 atomic mass units. A mass spectrometer does not measure mass directly but the *m/z* of the ions formed from the molecules. The charge on an ion is again conveniently represented in fundamental units, i.e. equal in magnitude to the charge on an electron and denoted by an integer number of z. Mass spectrometrists often speak loosely of the "mass of an ion" which is only strictly correct for singly charged ions. The different functional units of a mass spectrometer are represented in the following diagram (Figure 4.6).

Figure 4.6. Components of a mass spectrometer

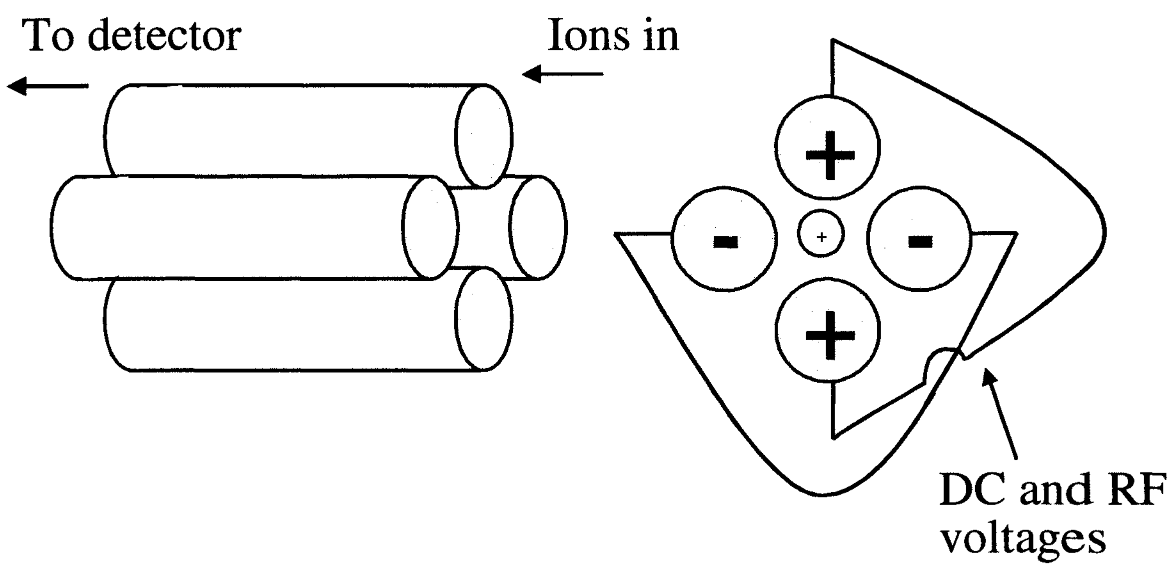


In terms of LC/MS the design of sources has already been discussed at great length. The next sections will look at the ion sorting or analysers available to the LC/MS user.

4.2.3 *QUADRUPOLE MASS ANALYSER*

As the name suggests this consists of four rods with opposite pairs connected electrically (see Figure 4.7). By applying voltages which consist of a radio frequency (RF) and direct current (DC) component it is possible to make the trajectory of a particular m/z stable and thus reach the detector. A mass spectrum is therefore produced by systematically altering the voltages in such a way that ions of increasing or decreasing m/z are detected. The quadrupole analyser is an ideal detector for chromatography as it is capable of fast scanning and uses low voltages, making it suitable for the relatively high operating pressure e.g. 10^{-6} Torr encountered in LC/MS [25]. The quadrupole is classified as a low resolution device, typically measuring m/z to the nearest integer value.

Figure 4.7. Schematic of a quadrupole mass analyser



Triple quapdrupole mass spectrometers are most widely used for selective and sensitive quantification of target compounds that show specific mass transitions in multiple-reaction monitoring (MRM). The hardware consists of three sets of quadrupoles in series but the second set (Q2) is not used as a mass separation device as it has only RF voltages applied. The RF-only quadrupole (or octapole) is used as a collision cell in tandem mass spectrometry (MS/MS) [20]. Q2 is also filled with a collision gas and in the majority of experiments a neutral gas, often a noble gas e.g. argon, is used. There are several modes of operation of a triple quadrupole instrument [26]:

Product ion scan: This is one of the most widespread scanning modes in MS/MS, also called "daughter ion scanning". Q1 is used to select an ion of interest which is provided with extra energy to induce fragmentation. The most common method used is to collide the ion with an inert gas in the reaction cell (Q2). The excited ions fall apart and product ions are scanned in Q3 to yield a product ion spectrum.

Precursor ion scan: In this case Q1 is operated in scan mode with Q3 fixed at a chosen m/z value. There is only a response at the detector when a precursor of the selected ion passes through Q1. This approach can be used to select a fragment typical of certain group with a compound. The resulting scan in Q1 yields a signal only for compounds bearing that group, thus reducing interference (chemical noise) of other compounds in the mixture.

Constant neutral loss: Both analysers are scanned with a fixed mass difference (Q1 higher than Q3). Only ions that lose that specific mass in the collision cell will be detected. This feature provides another opportunity to detect products of a particular class with similar structures.

Single/multiple reaction monitoring: The selectivity of mass selection can be used to enhance detection limits in analytical procedures. By fixing Q1 on the m/z ratio of interest, the signal at the detector is improved. This is simply explained by the fact that if Q1 were to be scanned an important fraction of ions of interest would be lost. A product ion characteristic of the analyte of interest is then selected in Q3 after fragmentation which will eliminate interference from isobaric ions.

Selection of two or more characteristic ions gives the term multiple reaction monitoring (MRM).

4.2.4 *QUADRUPOLE ION TRAP ANALYSER*

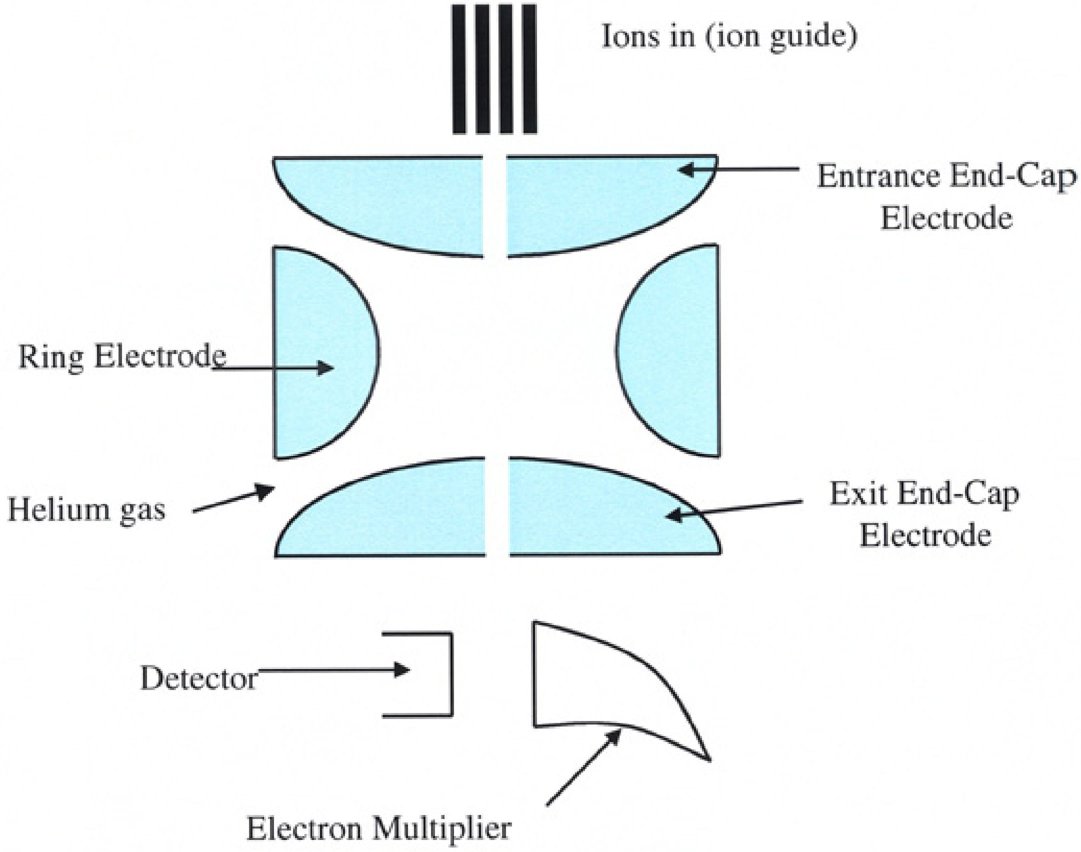
The quadrupole ion trap, usually referred to simply as an ion trap, is a three-dimensional quadrupole. It consists of a cylindrical ring electrode to which the quadrupole field is applied and two end-cap electrodes. This type of analyser is shown schematically in Figure 4.8. The top end-cap allows ions to pass in, ions are ejected towards the detector and out of the bottom end-cap. The ion trap differs from a quadrupole in that the mass analysis is a discontinuous pulsed process and scanning of the various m/z is based on ion instability. The reverse is true for a quadrupole [20]. The scanning process of an ion trap consists of the following steps:

- A pre-ionisation step determines the number of ions to be expected in the trap.
- An appropriate RF voltage is applied to the ring electrode and ions are injected in from the external ion source and stored there.
- At this stage all ions are held in stable but complex trajectory i.e. trapped, but a selected m/z can be ejected from the trap by modifying the RF voltage.
- In full-scan mode ions of different m/z are ejected by ramping the RF voltage at the ring electrode. Full-scan is a term used to describe where a mass spectrometer collects all ions present in a selected m/z range.

4.2.5 *TIME-OF-FLIGHT INSTRUMENTS*

In some respects, the time-of-flight (TOF) is the simplest of mass separation devices [25]. Ions produced in the source of an MS are given the same kinetic energy and velocity is inversely proportional to the square root of the mass. As a consequence of this the time taken to traverse the flight tube of the MS will be related to the m/z of the ion.

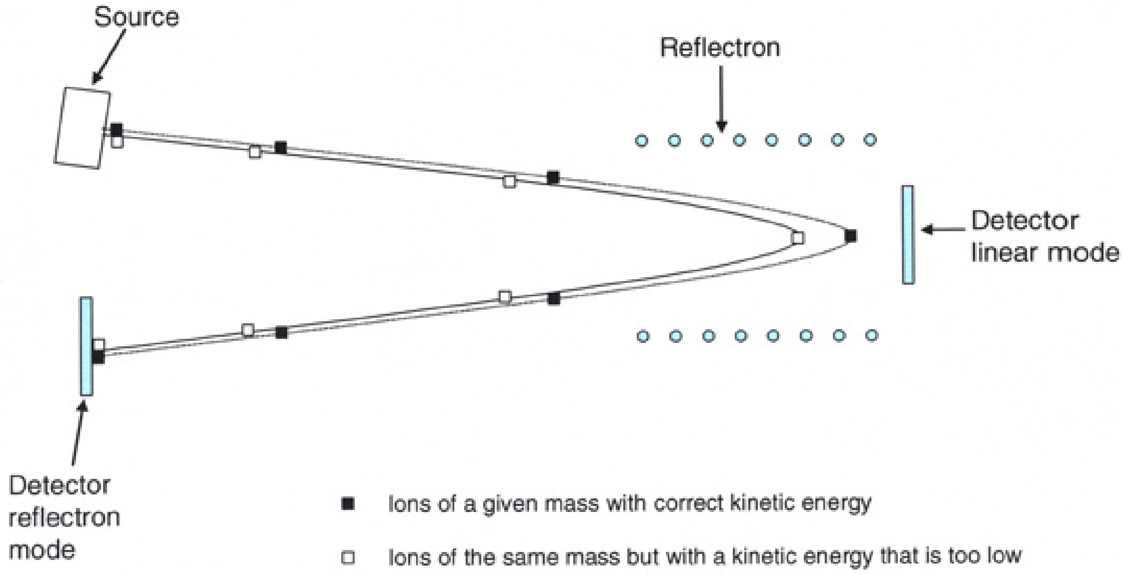
Figure 4.8. Quadrupole ion trap



In the first generation of TOF instruments ions passed directly from the source to the detector- termed linear TOFs. The most important drawback of the linear TOF analysers is their poor mass resolution. Mass resolution is affected by factors that produce a distribution in flight times of ions with the same m/z. These factors can include the length of ion formation pulse, the size of the volume where ions are formed and the variation in initial kinetic energy.

To increase the resolution of these analysers, one solution might be to increase the length of the flight tube. However a more practical approach to improve mass resolution is to use an electrostatic reflector, also called a reflectron. This creates a retarding field that acts as an ion mirror, deflecting ions back through the flight tube. The term reflectron TOF is used to differentiate it from a linear TOF. The reflectron corrects the energy dispersion of ions leaving the source with the same m/z ratio, as shown in Figure 4.9. In fact ions with more kinetic energy will penetrate the reflectron more deeply, spending more time in the reflectron. Hence they reach the detector at the same time as slower ions of the same *m/z*. The reflectron increases the mass resolution, but at the expense of sensitivity and the introduction of a mass range limitation [27]. Another advantage of TOF technology is the fast scanning capability which can be useful in fast analysis or when high chromatographic resolution is required [25].

Figure 4.9. Schematic diagram of a reflectron time-of-flight MS adapted from [27]



The principle of TOF-MS has been established for many years, but significant breakthroughs in the technique came about as a result of improved computer technology and the emergence of matrix assisted laser desorption ionisation (MALDI) [28]. Mass accuracies of better than 15 ppm (e.g. measured m/z = 100.0015, actual m/z = 100.0000) for routine reflectron instruments in combination with MALDI and better than 5 ppm for orthogonal acceleration TOF-MS in combination with ESI are routinely obtained [28]. The resolving power of the instruments may be sufficient to provide a molecular formula [29].

One way to perform MS/MS with a time-of-flight instrument is the creation of a hybrid instrument. The most successful of these is the quadrupole-time-of-flight (Q-TOF) [28]. This instrument provides accurate mass for product ions generated in MS/MS. Even more resolving power is provided by the Fourier transform ion cyclotron resonance (FTICR) mass spectrometers, but these instruments are normally too expensive for environmental application [29] and will not be discussed here. Similarly TOF mass spectrometers have to a large extent replaced high-resolution magnetic-sector double focussing instruments for LC/MS applications [26] and in the context of this work the latter will not be discussed.

4.2.6 ION DETECTION

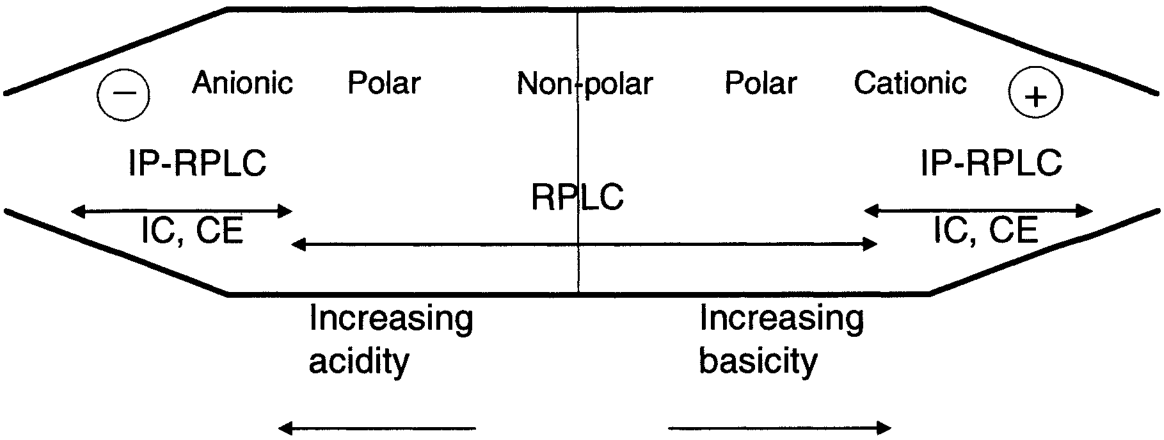
Most MS instruments are equipped with an electron multiplier for the detection of ions. There are discrete dynode types, where a series of around 15 beryllium-copper dynodes are electrically connected and continuous-dynode types, where a continuous resistive strip is coated on glass. In an electron multiplier an ion beam is converted to an electron beam and the signal is amplified by a cascade effect. Since the first dynode is normally at a potential of -1 to -3 kV, the electron multiplier cannot be used directly for the detection of negative ions. To solve this problem a conversion dynode at typically +5 kV, which on contact with negative ions produces electrons, is placed in front of the electron multiplier.

4.3 Liquid Chromatography

4.3.1 INTRODUCTION

This section will cover the aspects of liquid chromatography (LC) considered most important for the LC/MS user and those most relevant for the context of this work. Appropriate books are available covering LC method development in more detail e.g. Synder [30]. Mass spectrometry (MS) is becoming the detection system of choice for LC analysis in many areas [31]. Methods sometimes need to be translated from LC-ultraviolet (UV) detection methods because additives present in the LC-UV mobile phases have a negative effect on MS ionisation, performance and maintenance [30]. The high selectivity of MS has allowed shorter columns and runtimes, thus enabling high throughput screening to take place. Since the physico-chemical properties of environmental contaminants like molecular mass, acidity/basicity and hydrophobicity cover a wide range of values, different analytical techniques are required to meet these challenges [26]. However reversed–phase liquid chromatography (RPLC) is by far the most widely used chromatographic technique in LC/MS. The diagram in Figure 4.10, adapted from [29], gives an overview of chromatographic techniques as applied to LC/MS.

Figure 4.10 Interrelationship between analyte properties and the appropriate chromatographic separation method [29].



4.3.2 REVERSED-PHASE LC

The most important and widely used separation technique for environmental applications in aqueous samples is reversed-phase LC. The stationary phase in RPLC consists of a non-polar matrix while the mobile phase is a polar solvent. In practice, relatively hydrophilic compounds elute earlier with the aqueous mobile phase, consisting of water mixed with a polar organic modifier (e.g. methanol or acetonitrile). Increasing the strength of the organic modifier in the mobile phase elutes the more strongly retained hydrophobic material earlier. Typically, octadecyl (C_{18})-bonded silica and octyl (C_8)-bonded silica are the standard separation materials. The chemistry of the bonded phase and shielding of residual silanols determine the selectivity and applicability of a column [26].

The use of LC/MS in water analysis is directed towards polar compounds and the proportion of water in the elution system is often high. Retention of weakly acidic or basic compounds may be improved by adding a volatile acid or base (e.g. ammonium acetate) [28]. For very acidic or basic compounds the formation of ion-pairs is often desirable to increase retention in RPLC [29].

4.3.3 COMBINING LC WITH API-MS

In general, API techniques require the use of volatile solvent additives, listed in Table 4.1, to prevent source contamination or plugging of the sample orifice. In particular phosphate, sulphate and borate additives commonly employed in LC are not suitable for API-MS.

Table 4.1. Suitable solvents and additives for API-MS [11]

Parameter	Solvents/additive
pH	acetic acid, formic acid, trifluoroacetic acid (TFA), for ESI positive-ion detection; ammonium hydroxide for ESI negative-ion detection (typically 0.1-1% range)
Buffers, ion pair reagents	Ammonium acetate, ammonium formate, triethylamine, heptafluorobutyric acid (HFBA), tetraethyl or tetrabutylammonium hydroxide (TEAH or TBAH) 10–100 mM level)
Cationisation reagents	Potassium or sodium acetate (20-50 μM level)
Solvents for API-MS	Methanol, ethanol, propanol[b], isopropanol[b], butanol[b], acetonitrile, water, acetic acid[a], formic acid[a], acetone, dimethylformamide[a], dimethylsulphoxide[a], 2-methoxy ethanol[b], tetrahydrofuran[a], chloroform[a]
Solvents for APCI only (not suitable for ESI)	Hydrocarbon solvents (e.g. hexane, cyclohexane, toluene), CS_2, CCl_4

a solvents tested in the 5-20 % range

b good for negative-ion operation

In general compounds with basic sites (e.g. amines) should be analysed at low pH using positive-ion detection. Components containing acidic sites (e.g. carboxylic acids) should be analysed at high pH using negative-ion detection. Neutral species can be analysed in ESI mode by the addition of micromolar concentrations of cationisation agents such a sodium and potassium acetate [11].

4.4 Conclusion

This chapter has covered the basics of LC and MS and their combination in the hyphenated technique of LC/MS. A brief overview of the development of LC/MS has also been included, up to the present position where the reliability and acceptance of electrospray has meant its wide use in a lot of practical areas. A more detailed discussion of LC/MS in respect to alcohol ethoxylates (AEs) and practical method development of electrospray for the analysis of AEs will follow in Chapter 6.

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AIMS

Current literature methodology is unable to quantitatively extract and determine alcohol ethoxylates (AEs) in environmental waters, soils and sediments reflecting the true distribution of these species. This is commonly known as obtaining a "fingerprint" of the AE distribution in the environment. This project will address the shortcomings of the existing methodologies and build on previous work done by Unilever Research and the University of Northumbria in the quantitation of AEs in the environment. The project will use commercially available AE samples obtained from Clariant and BASF, which demonstrate a C_{12} to C_{18}, EO_{0-20} distribution. These materials are representative of those used by industry, and are in line with an industrial Task Force, Environmental Risk Assessment and Management (ERASM) study, looking at the AE fingerprint in the environment.

Initially analyte derivatisation with phthalic anyhydride will be used in conjunction with LC/MS, to increase sensitivity for "total AE" and in turn increase selectivity for individual ethoxymers. Reflecting the actual AE distribution of the commercial samples, which has been previously identified by NMR will be key to success here. Extraction methods from both aqueous and solid samples will be optimised to allow the AEs to be analysed in all environmental compartments. Due to the complex and diverse physical chemical properties of AE compounds, features which give useful detergency properties, considerations have to be made for extraction losses due to volatility, polarity and hydrophobicity. This study attempts to maximise recovery for all 126 compounds present in the Commercial samples tested. Finally the optimised methodology will be used to elucidate mechanisms involved in AE biodegradation.

Chapter 5

EXPERIMENTAL SECTION

5.1 Chemicals and reagents

5.1.1 SOLVENTS

Solvents (methanol, methyl tertiary-butyl ether (MTBE), dichloromethane (DCM), acetonitrile (ACN), tetrahydrofuran (THF) and pyridine) of LC quality were obtained from Rathburn Chemicals Ltd. (Walkerburn, UK). Phthalic anhydride (>99 %) was obtained from Fisher Scientific (Loughborough, UK) and ammonium hydroxide (33%, extra pure) was obtained from Fluka Ltd. (Gillingham, UK). The Ultrapure water used in the preparation of mobile phases was Milli Q Plus (Millipore, Watford, UK).

5.1.2 COMMERCIAL SAMPLES

Three commercial alcohol ethoxylates (AEs) were used. These were Genapol C100 (a linear AE with C_{12} and C_{14} alkyl chains and an average of 10 ethoxy (EO) units), Genapol T110 (a linear AE with C_{16} and C_{18} alkyl chains and average of 11 EO units), both supplied by Clariant (Frankfurt, Germany). Lutensol A07 (a linear AE with C_{13} and C_{15} alkyl chains and average of 7 EOs) was supplied by BASF (Ludwigshafen, Germany). Equal amounts were dissolved in either methanol or pyridine for spiking or calibration standards, respectively.

5.1.3 STANDARD PREPARATION

An internal standard, n-hexadecyl-d_{33} alcohol, from QMx (Thaxted, UK) was prepared at 1000 μg/mL in pyridine. Equal weights of Genapol C100, Genapol T110 and Lutensol A07 were dissolved in pyridine or methanol to give 6000 μg/mL total AE stock solutions. The methanol stock was then serially diluted as required to prepare spiking solutions in methanol or acetone. Derivatisation reagent was prepared by dissolving 3.0 g of phthalic anhydride in 50 mL of pyridine using ultrasonication. The reagent was stable for 1 month at 4 °C in the dark. The pyridine stock was diluted and derivatised in an oven at 85 °C for 1 hr to give AE derivatives of concentrations 0, 7.5, 15, 30, 60, 120 μg/mL.

5.1.4 *INDIVIDUAL ETHOXYLATES*

Individual ethoxylates (EOs) and alcohols, purity >98%, were supplied by Fluka Ltd. (Gillingham, UK) as follows: 1 dodecanol ($C_{12}EO_0$), 1 tetradecanol ($C_{14}EO_0$),1 hexadecanol ($C_{16}EO_0$), 1 octadecanol ($C_{18}EO_0$), diethylene glycol monododecyl ether ($C_{12}EO_2$), diethylene glycol monotetradecyl ether ($C_{14}EO_2$), diethylene glycol monohexadecyl ether ($C_{16}EO_2$), octaethylene glycol monododecyl ether($C_{12}EO_8$) octaethylene glycol monotetradecyl ether ($C_{14}EO_8$),octaethylene glycol monohexadecyl ether ($C_{16}EO_8$) and octaethylene glycol monooctadecyl ether ($C_{18}EO_8$). Each ethoxylate was prepared as a 1 μM mix in either acetone or methanol for spiking purposes, and in pyridine for calibration standards.

5.2 Apparatus

The samples and standards were analysed on an 1100 LC/MS from Agilent Technologies Ltd, (Stockport, UK). The system comprised of a G1322A Solvent degasser, G1313A autosampler, G1312A HPLC pump, G1316A column module and G1946B version MSD.

The syringe pump used was a Pump 11 from Harvard Apparatus Ltd. (Kent, UK). The autotrace SPE Workstation was from Zymark Ltd. (Runcorn, UK). The Reacti-vap model 18780, used for the concentration of sample extracts, was from Pierce and Warriner (Chester, UK). Screw top vials with PTFE inserts (18 mL), used in SPE procedures, were obtained from Fisher Scientific (Loughborough, UK).

Chromatographic separation was carried out on a variety of analytical columns. Due to the high pH of the mobile phase, columns offering stability in those conditions such as a Luna C_{18} (2) analytical column (150 x 2 mm ID, 5 μm particle size) from Phenomenex, (Macclesfield, UK) or a Pursuit C_{18} (150 x 2.1 mm ID, 5 μm particle size) from Varian (Richmond on Thames, UK) were used. A 2 mm id C_{18} guard cartridge of equivalent phase to the column was also used.

5.3 Extraction procedures

5.3.1 AUTOMATED SPE

SPE was carried out on Isolute C_8 cartridges (1 g, 6 mL) from Argonaut (Hengoed, UK). The procedure was automated, using the autotrace SPE Workstation (see Section 5.2). The cartridges were conditioned with 10 mL elution solvent (methanol : MTBE : DCM, 2:1:1, v/v/v), followed by 10 mL of methanol and 10 mL of Ultrapure water. The samples were loaded at 10 mL/min, and dried for 60 min under nitrogen before being eluted with 15 mL of elution solvent. The extracts were initially concentrated to 1-2 mL under a gentle stream of nitrogen on a Reacti-vap unit (see Section 5.2), at room temperature, before being quantitatively transferred to an HPLC vial and carefully taken to dryness prior to derivatisation.

5.3.2 MANUAL SPE

Procedures were followed as in Section 5.3.1 but cartridges were conditioned, loaded and dried on a Vac Elut vacuum manifold. The nitrogen drying step was replaced by pulling air through the cartridge with a vacuum pump.

5.4 Derivatisation

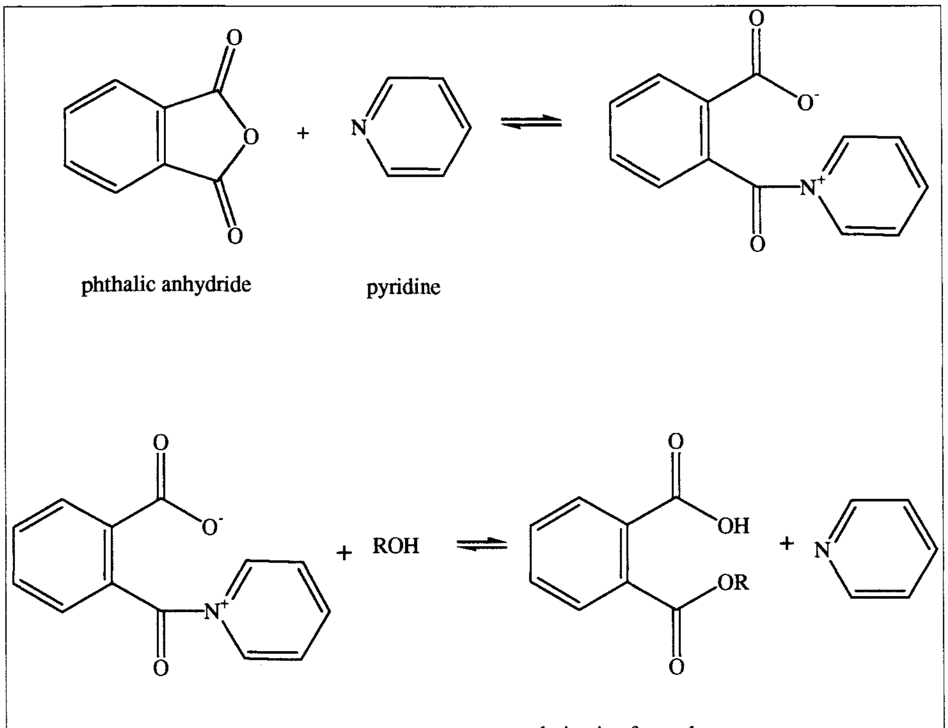
5.4.1 PROCEDURE FOR SAMPLES

Samples were resuspended in 990 μL of a phthalic anhydride in pyridine solution (Section 5.1.3) and 10 μL of internal standard (Section 5.1.3) were added. The samples were then capped and heated at 85°C for 1 hour in an oven for derivatisation to occur.

5.4.2 DERIVATISATION REACTION

The mechanism for the reaction is shown in Figure 5.1.

Figure 5.1 Phthalic anhydride derivatisation of alcohols (reproduced from Goss[1])



R in Figure 5.1 is of the form $R^1(OCH_2CH_2)_n$, where R^1 is a C_{12} to C_{18} alkyl group and n = 0 to 20

5.5 LC/MS analysis

5.5.1 FULL SCAN METHOD

Samples were analysed on an Agilent 1100 LC/MS (see section 5.2). AEs were separated under gradient elution conditions with a mobile phase of A, 0.1 % v/v ammonia in Ultrapure water (v/v) and B, 0.1 % v/v ammonia in 900/100 (v/v) ACN/THF. The mobile phase composition was 15 % B at the beginning of the gradient and then linearly increased to 100 % B in 25 min. It was then held for a further 10 min before re-equilibrating at the initial conditions for 20 min. The AE derivatives were separated on a C_{18} analytical column (150 mm x 2 mm i.d., 5 μm particle size) and 2 mm i.d. C_{18} guard cartridge (see section 5.2). The flow rate was maintained at 0.25 mL/min and 10 μL of the pyridine extracts were injected onto the column which was thermostatically held at 40 °C. A divert time of 8 min was used to minimise source contamination from the derivatisation reagent.

The MS was operated in negative ion mode electrospray with a gas temperature of 300 °C, drying gas 7.0 L/min, nebuliser gas pressure 35 psi, and a capillary voltage of 4000 V. The MS was operated in full scan mode over the range m/z 300 to 1300 and a fragmentor voltage ramp (typically from 84 to 256 V over the range m/z 333 to 1214) was utilised to give optimal performance. A list of ions analysed by the LC/MS, as extracted ions from the full scan data, are shown in Chapter 6 (Table 6.5.)

5.5.2 SINGLE ION MONITORING (SIM) FOR C_{12} AND C_{18} AEs

A single ion monitoring (SIM) method was also developed to give more sensitivity in the analysis of selected AEs. The conditions of the method were essentially the same as in Section 5.5.1., however in this case ions for C_{12} and C_{18} AEs (see Table 6.5) were selectively monitored in two groups and set up according to the retention times of the alkyl chain homologues. This approach could not be used for the full suite of AEs due to chromatographic overlap of homologues and the limiting number of ions that could be included in a SIM window on the Agilent system.

Reference List

[1] J.Goss, Chromatographia **38** (1994) 417.

DEVELOPMENT OF THE PHTHALIC ANHYDRIDE DERIVATISATION LC/MS METHOD FOR THE ANALYSIS OF AEs

6.1 Introduction

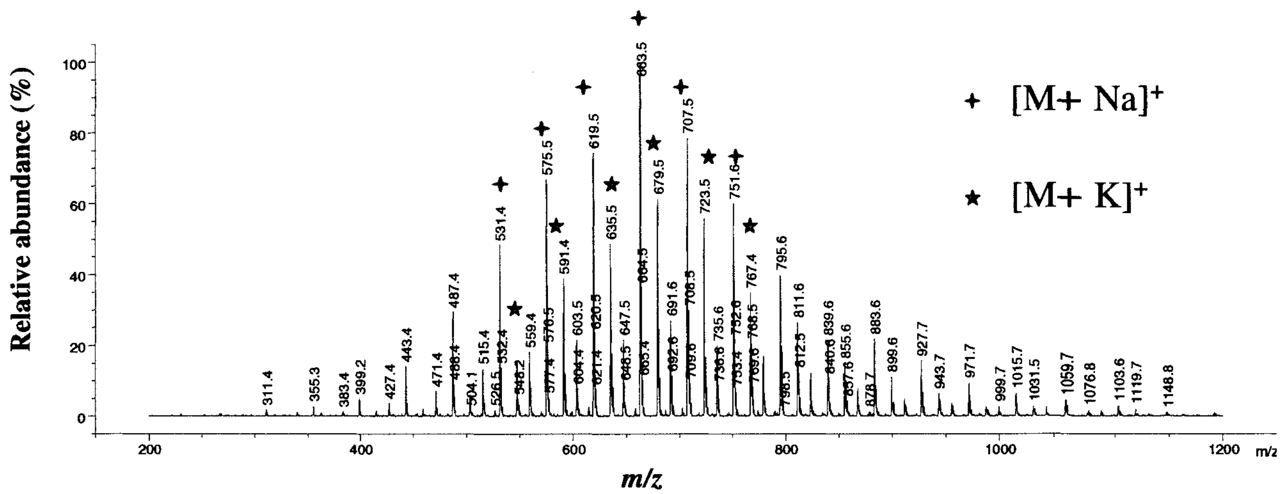
The reason for choosing liquid chromatography/mass spectrometry (LC/MS) for the analysis of alcohol ethoxylates (AEs) is that it offers a flexible and efficient chromatographic separation of the complex mixtures, spanning a wide range of molecular mass, polarity and volatility. MS offers high sensitivity and selectivity which are required in the examination of challenging matrices. Environmental analysis is normally performed at trace concentrations with limited sample amounts that have been pre-concentrated, as selective clean-up maybe difficult. The detection system also allows for structural elucidation of the complicated species, whilst at the same time enabling accurate quantitation. Importantly the LC separation may not have to be too sophisticated as extracted ions on the LC/MS can be used to resolve co-eluting or partially co-eluting species.

It is well documented that AEs in positive ionisation mode will form adducts of the type $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$[1,2]. The extent at which the different adducts form depends on the reagents used and also on impurities within the samples. A study on the chromatographic behaviour in atmospheric pressure chemical ionisation (APCI) mode by Jandera et al. [1] demonstrated the formation of these types of adducts. Similarly in electrospray ionisation (ESI) mode Sherrard et al. [2], while investigating AEs used in wool scouring, noticed similar mixed adduct formation. However by the addition of an excess of NaCl they were able to force the adducts to $[M+Na]^+$, thereby simplifying the spectra. There is however no evidence in the literature that the free alcohol and lower ethoxylates (EOs) ionise very efficiently by either ESI or APCI. In a review on the characterisation of surfactants [3], Di Corcia states that a weak signal was obtained for EO_1 but the response by ESI-MS increased exponentially for EO_{1-6}. An effect which was explained by the ability of molecular species to form adducts with cations in solution, being influenced by the number of polar sites in the molecule, and the fact that polyethoxylate species can form increasingly stable complexes with cations as EO number increases. As discussed in Section 3.1, the AE molecule does not possess a chromophore, requiring derivatisation when analysed by conventional LC detectors. The move to LC/MS, as AE method development evolved, was designed to eliminate the need for derivatisation in the analysis. However, derivatisation in combination with LC/MS will be investigated to analyse the entire range of AE ethoxymers within the samples studied. One such method by Dunphy et al. [4], imparted a permanent cationic charge on the AEs using 2-fluoro-N-methylpyridinium p-toluenesulphonate. However problems included the compatibility of the cationic derivatives with silica based LC separations which required basic modifiers such as triethylamine (TEA), leading to MS signal suppression. On solving this problem using polymeric HPLC phases, there were still reported inteferences, particularly in the lower ethoxylates, requiring MS/MS analysis. An alternative approach will be investigated by preparing phthalic anhydride derivatives of the AE materials. Initial method development examined the ionisation of unaltered AEs in comparison with derivatisation. Techniques that bypass the LC separation such as flow injection analysis (FIA) and syringe infusion were first of all used to optimise MS conditions.

6.2 Underivatised AE

Lutensol A07, diluted in methanol (392 µg/mL) was sprayed directly into an ESI source using syringe infusion. The infusion rate was 200 µL/min and the ESI was operated in positive ionisation mode with source conditions as follows: nebuliser 35 psi, gas flow 7 L/min, gas temperature 250 °C and capillary voltage (V_{cap}) of 3000 V. The ion formation was shown to be a mixed adduct with $[M+Na]^+$ and $[M+K]^+$ predominating. The spectrum shown in Figure 6.1 is characteristic of an ethoxylated surfactant with a series of ions 44 *m/z* apart, representing molecules with differing numbers of CH_2CH_2O groups. The series of ions with *m/z* values of 531.4, 575.4, 619.5 and 663.5 are the sodium adducts for $C_{13}EO_7$ to $C_{13}EO_{10}$, respectively (Table 6.1). The series of ions with *m/z* values of 591.4, 635.5, 679.5 and 723.5 are the potassium adducts for $C_{13}EO_8$ to $C_{13}EO_{11}$, respectively. Formation of ions of this type from a methanolic solution are due to the high availability of Na^+ and K^+ ions from organic solvents [3] and glassware [5]. There is however no ionisation of the lower ethoxylates $C_{13}EO_{0-1}$, with $C_{13}EO_{1-2}$ *m/z* 311.3 and 355.3 showing weak ionisation. This type of ionisation, relying on adduct formation from potentially varying amounts of metal ions, can also prove to be problematical from an analytical perspective as varying ratios of the ions of differing adducts in samples and standards can lead to errors in quantitation. In practice it is better to force adducts of one type, for example by addition of acid or ammonia to give $[M+H]^+$ or $[M+NH_4]^+$ ions, respectively.

Figure 6.1 Mixed adduct formation of underivatised Lutensol A07 infused in methanol only



6.3 Preparation of a phthalic anhydride derivative

The first attempts to facilitate the ionisation of the AEs by providing a phthalic anhydride derivative, were also carried out by syringe infusion and flow injection analysis (FIA). FIA is where an injection is made directly into the flow of mobile phase. The composition of mobile phase for these experiments was that previously used by other workers within Unilever for the LC separation of the derivatives. A typical mobile phase contained water/acetonitrile (ACN)/tetrahydrofuran (THF)/ammonia (500/450/50/1, v/v). Positive ionisation had also been used with the AEs forming ammonium adducts. A typical experiment consisted of a 1 μL injection of a Genapol C100 derivative (1 mg in 5 mL) into a flow of mobile phase at 1 mL/min. This however resulted in the formation of predominantly sodium adducts, despite increasing the ammonia concentration in the mobile phase to 0.5 %

v/v. This was not a desirable outcome because sodium adducts do not give good fragmentation data in MS/MS [5] if the method was adapted in that way. Variation of the fragmentor voltage allowed some optimisation of the $[M+NH_4]^+$ adduct but $[M+Na]^+$ was still the major signal.

Table 6.1 Possible adduct formation for an underivatised solution of Lutensol A07 (*m/z*)

Ethoxylate	NH_4^+	H^+	Na^+	K^+
$C_{13}EO_0$	218.3	201.2	223.2	239.2
$C_{13}EO_1$	262.3	245.3	267.2	283.2
$C_{13}EO_2$	306.3	289.3	311.3	327.2
$C_{13}EO_3$	350.4	333.3	355.3	371.3
$C_{13}EO_4$	394.4	377.4	399.3	415.3
$C_{13}EO_5$	438.4	421.4	443.4	459.3
$C_{13}EO_6$	482.4	465.4	487.4	503.4
$C_{13}EO_7$	526.5	509.4	531.4	547.4
$C_{13}EO_8$	570.5	553.5	575.5	591.4
$C_{13}EO_9$	614.5	597.5	619.5	635.5
$C_{13}EO_{10}$	658.6	641.5	663.5	679.5
$C_{13}EO_{11}$	702.6	685.6	707.6	723.5
$C_{13}EO_{12}$	746.6	729.6	751.6	767.6
$C_{13}EO_{13}$	790.7	773.6	795.6	811.6
$C_{13}EO_{14}$	834.7	817.7	839.6	855.6
$C_{13}EO_{15}$	878.7	861.7	883.7	899.6
$C_{13}EO_{16}$	922.8	905.7	927.7	943.7
$C_{13}EO_{17}$	966.8	949.8	971.7	987.7
$C_{13}EO_{18}$	1010.8	993.8	1015.8	1031.7
$C_{13}EO_{19}$	1054.9	1037.8	1059.8	1075.8
$C_{13}EO_{20}$	1098.9	1081.9	1103.8	1119.8

The sodium ion formation was thought most likely to be due to the presence of sodium impurities in reagents. An experiment was devised to provide a crude LC separation of the derivative mixture prior to introduction into the MS to observe the ion formation in the situation where the analytes or AEs were separated from the excess derivatisation reagent and solvents. An isocratic mobile phase was run at 1 mL/min containing the reagents and proportions listed above through a Hypersil BDS C_8 5 μm 250 x 4.6 mm column. Under these conditions the C_{12} and C_{14} eluted at approximately 4 and 7 min, respectively. The conditions and exact details of this method are not critical but the fact that the resulting mass spectrum of each peak now showed the ammonium adduct formation was the desired outcome of the experiment. This suggested that separation on the column removed the excess of

Na^+ ions from possible interference with eluted derivatives. It also followed that the source of the Na^+ was in the derivative solution. The initial work did not however appear to improve the ionisation of EO_{0-2} AE which was the main objective of the derivatisation. However using the findings from this work and also the need to get syringe infusion or FIA to work to facilitate easier optimisation of MS conditions the following approach was made:

1) A concentrated derivative was prepared which was diluted with mobile phase thus reducing the effect of derivative reagents.
2) A simplified derivatised AE mix was also be prepared from individual C_{12} AEs. One of the key reasons for doing this was to target the free alcohol ionisation.

6.4 Individual EO mix

Individual ethoxylates had been previously synthesised at Unilever. These included hexaethyleneglycol monodecylether ($C_{12}EO_6$, purity 100.0 % w/w), dodecylethyleneglycol monodecylether ($C_{12}EO_{12}$, purity 97.2 % w/w) and eicosaethyleneglycol monodecylether ($C_{12}EO_{20}$, purity 99.4 % w/w). 1-dodecanol ($C_{12}EO_0$) was as described in Section 5.1.4.

A C_{12} mix containing only the four components listed above was used to simplify the optimisation process. A concentrated derivative was prepared and diluted in mobile phase as described. $C_{12}EO_0$ (48.6 mg), $C_{12}EO_6$ (53.9 mg), $C_{12}EO_{12}$ (46.9 mg) and $C_{12}EO_{20}$ (47.7 mg) were weighed directly into a screw top glass vial. To this phthalic anhydride (0.30 g) and pyridine (5 mL) were added before placing in an oven at 85°C for 1 hour. 100 µL of the resultant solution was added to a 10 mL volumetric and made up to volume with mobile phase (500 mL Ultrapure water, 450 mL ACN, 50 mL THF and 3 mL 33% ammonia solution). This gave the following concentrations $C_{12}EO_0$ (97.2 µg/ml), $C_{12}EO_6$ (107 µg/ml), $C_{12}EO_{12}$ (93.7 µg/ml) and $C_{12}EO_{20}$ (95.4 µg/ml).

The diluted solution was put in a 1 mL syringe and infused at 50 µL/min into the MS using a syringe pump (Section 5.2). Typical MS conditions for syringe infusion were as follows. Drying gas 12.0 L/min, gas temperature 350 °C, nebuliser 30 psi and multiplier gain of 5. Positive and negative ionisation modes were used.

The results of this work provided the following key results. The dilution into mobile phase gave the desired ammonium adduct formation. In Figure 6.2, ions at *m/z* 616.6, 880.6 and 1232.8 were the expected $[M+NH_4]^+$ adducts for EO_6, EO_{12}, and EO_{20}, respectively (M is the phthalic anhydride derivative). The ion seen at *m/z* 625.5 is the $[M+ 2NH_4]^{2+}$ adduct for EO_{20}. However despite varying fragmentor voltages, and using the software fragmentor optimisation ramps, no response could be seen for the EO_0 (free alcohol).

The same solution was reanalysed in negative ion ESI. At this point a breakthrough occurred in that four very intense peaks were obtained, $[M-H]^-$ ions. The negative ion had never been observed in pyridine solutions by FIA. The large dilution into mobile phase had obviously helped its formation. Theoretically with the derivative formed and pH of the mobile phase (>10) this was always expected (loss of H from COOH group. Note the much cleaner spectrum in negative ion mode (Figure 6.3). The ions at *m/z* 333.2, 597.4, 861.5 and 1213.8 represent the $[M-H]^-$ ions for EO_0, EO_6, EO_{12}, and EO_{20}, respectively. Significantly a good response for the EO_0 derivative, the C_{12} free alcohol was also obtained. These ions were also optimised using the fragmentor ramp feature in the Agilent Chemstation software. The fragmentor was increased linearly from 84 to 256 V over the range *m/z* 333 to 1214. The response of each AE appeared in the right order of magnitude as an equal concentration mix was used. Figure 6.4 indicates the structures of the ions formed in positive and negative ESI for the $C_{12}EO_6$ derivative.

Figure 6.2 Positive ion ESI, four component C_{12} mix

Syringe infusion at 50 µL/min of approximately 100 µg/mL each component in mobile phase.

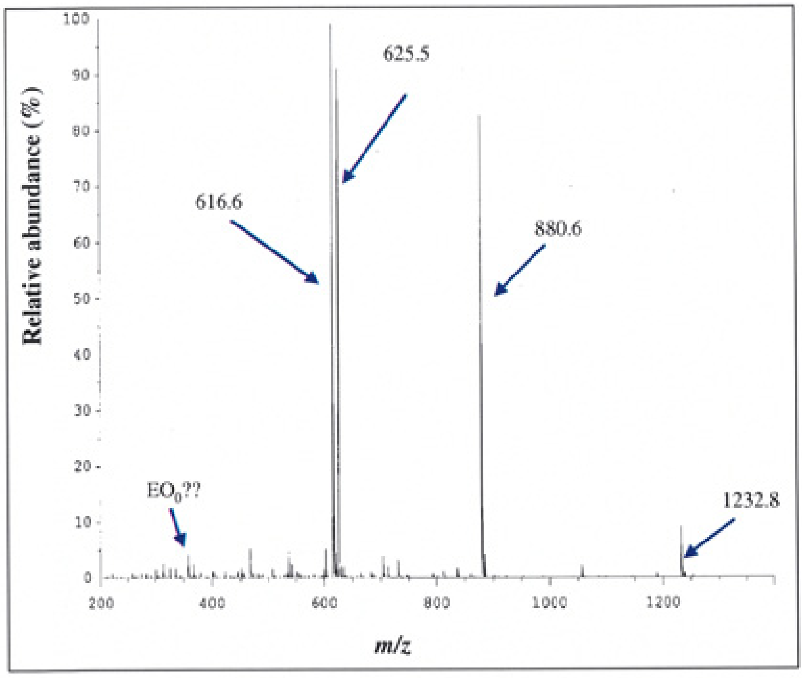


Figure 6.3 Negative ion ESI, four component C_{12} mix

Syringe infusion at 50 µL/min of approximately 100 µg/mL each component in mobile phase

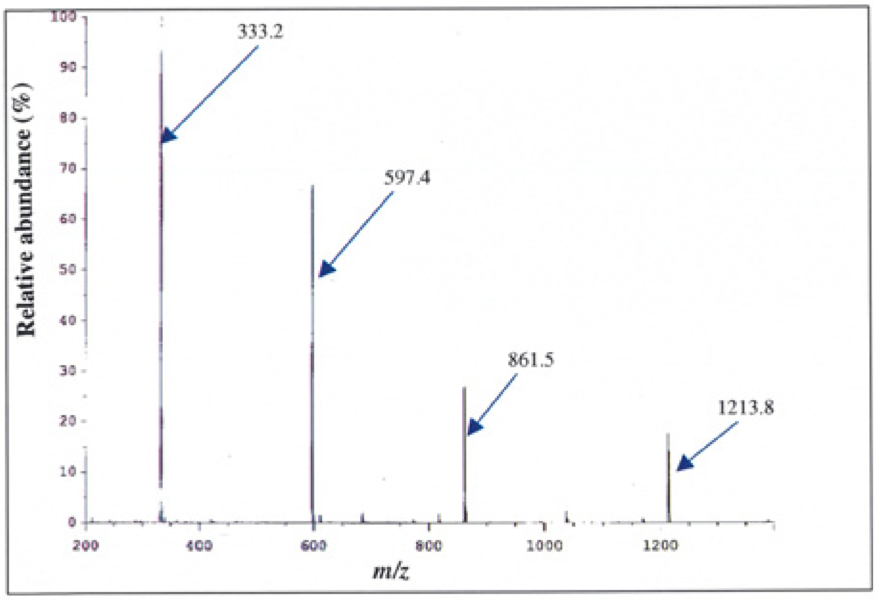
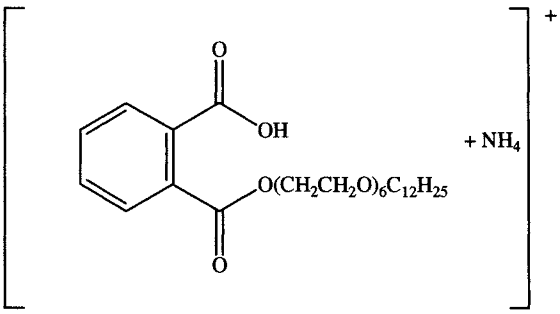
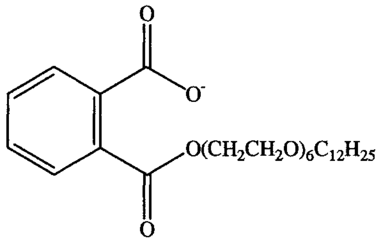


Figure 6.4 Structures of ions observed for derivatives of $C_{12}EO_6$ in positive and negative ESI



Exact Mass: 598.4 + 18.0 = 616.4



$C_{32}H_{53}O_{10}^-$
Exact Mass: 597.4

6.5 Infusion of commercial standards

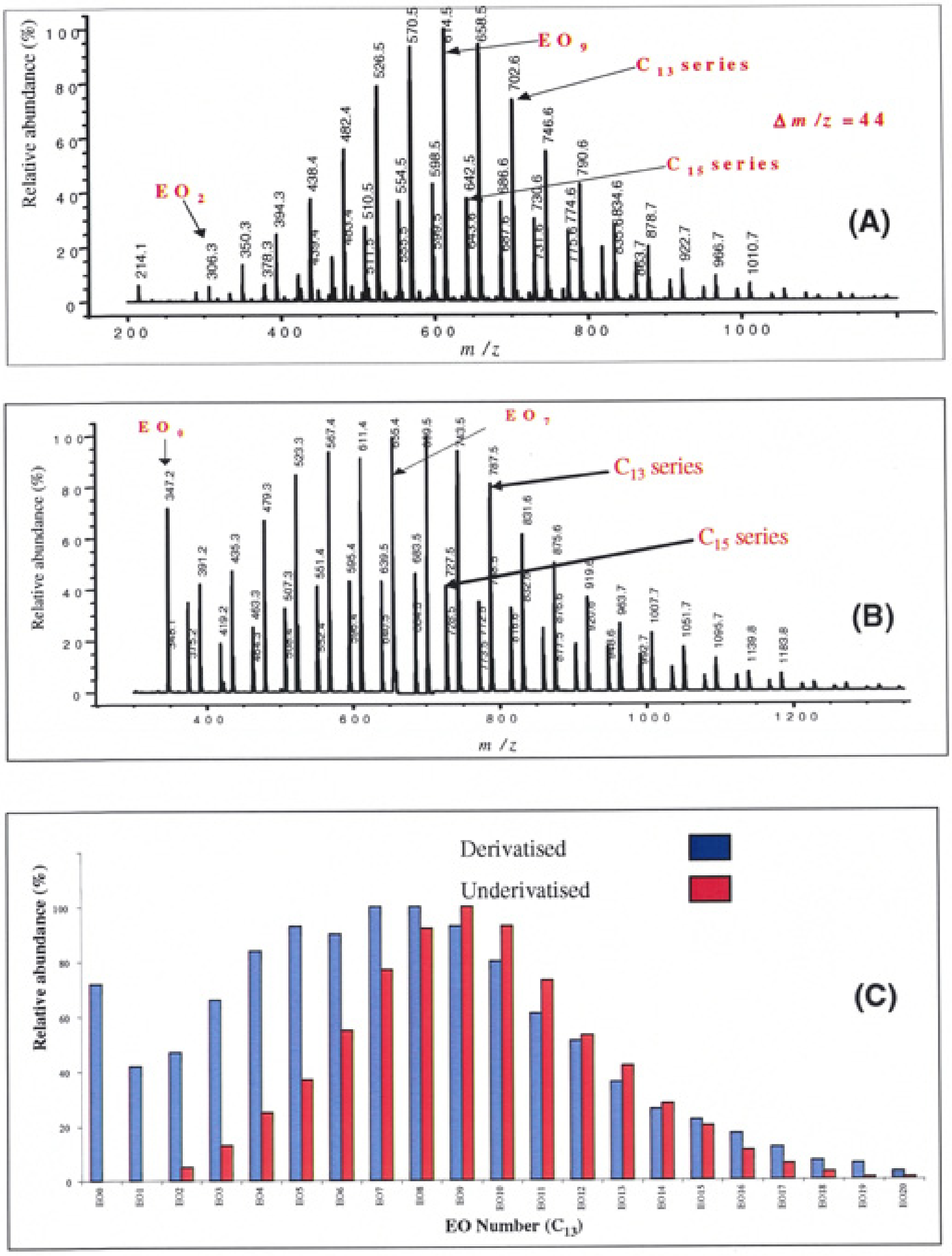
6.5.1 LUTENSOL A07 DERIVATISED AND UNDERIVATISED

The dilution of a concentrated derivative into mobile phase had allowed syringe infusion in negative ion mode to work. Although not tried at this stage FIA of the same diluted derivative could also be used. This represents an important finding and one which workers trying to reproduce this methodology should be aware of, as FIA or syringe infusion are inevitably the first steps used in transfer of a method to another instrument. The same approach could now be applied to commercial standards to show the benefits of ionisation of a derivative compared to existing methodology with an underivatised AE.

Negative ion ESI-MS of a derivative was compared to a positive ion ESI of an underivatised material. Negative ion ESI-MS response was optimised by syringe infusion of a concentrated Lutensol A07 derivative (200 mg / 5 mL derivatisation reagent) diluted 100-fold with mobile phase (50 : 50, v/v, A : B, Section 5.5.1). A similar concentration of Lutensol A07, to that of the diluted derivative, was also infused in the same mobile phase mixture in an underivatised form to show the benefit imparted by the derivatisation process. The results of these experiments are shown in Figure 6.5. Without derivatisation there is no apparent ionisation of EO_0 or EO_1 for either the C_{13} or C_{15} alkyl series (Figure 6.5(A)). The first peak visible in the C_{13} series, characterised by a difference in *m/z* of 44 units of the $[M+NH_4]^+$ adducts, is the *m/z* 306.3 ion which corresponds to $[C_{13}EO_2+NH_4]^+$. Ionisation of EO_0 and EO_1 would give ions of *m/z* 218 and 262, respectively. The EO distribution maximises at approximately $C_{13}EO_9$, *m/z* 614.5 (Figure 6.5 (A)). However for the derivatised sample the $C_{13}EO_0$ is clearly visible at *m/z* 347.2 (Figure 6.5(B)). This peak corresponds to the C_{13} alcohol phthalic anhydride derivative with the loss of a proton, producing a negative ion. The EO distribution maximises at approximately $C_{13}EO_7$ (Figure 6.5 (B)), which is in agreement with NMR data (Section 6.10). Figure 6.5 (C) shows a graphical comparison of the underivatised and derivatised 'fingerprint' forms of the AE. It can be clearly seen that with derivatisation a shift of MS response to lower EO distribution is made which is in keeping with theory and supporting NMR data. The derivatisation should therefore allow a more accurate environmental fingerprint of an AE to be established. This is very important in allowing shifts in chemical composition found in environmental monitoring to be incorporated into risk assessment data obtained from testing of parent material in laboratory toxicity studies [6]. In addition it is noted that the spectrum (Figure 6.5 (C)) is largely free from background interference when observed in negative ion mode, with ionisation of the derivatives not subject to competition from other adducts or doubly charged species as can occur in positive ESI data.

Figure 6.5 Syringe infusion of Lutensol A07. (A) underivatised form; (B) derivatised form; and, (C) graphical comparison of underivatised and derivatised forms.

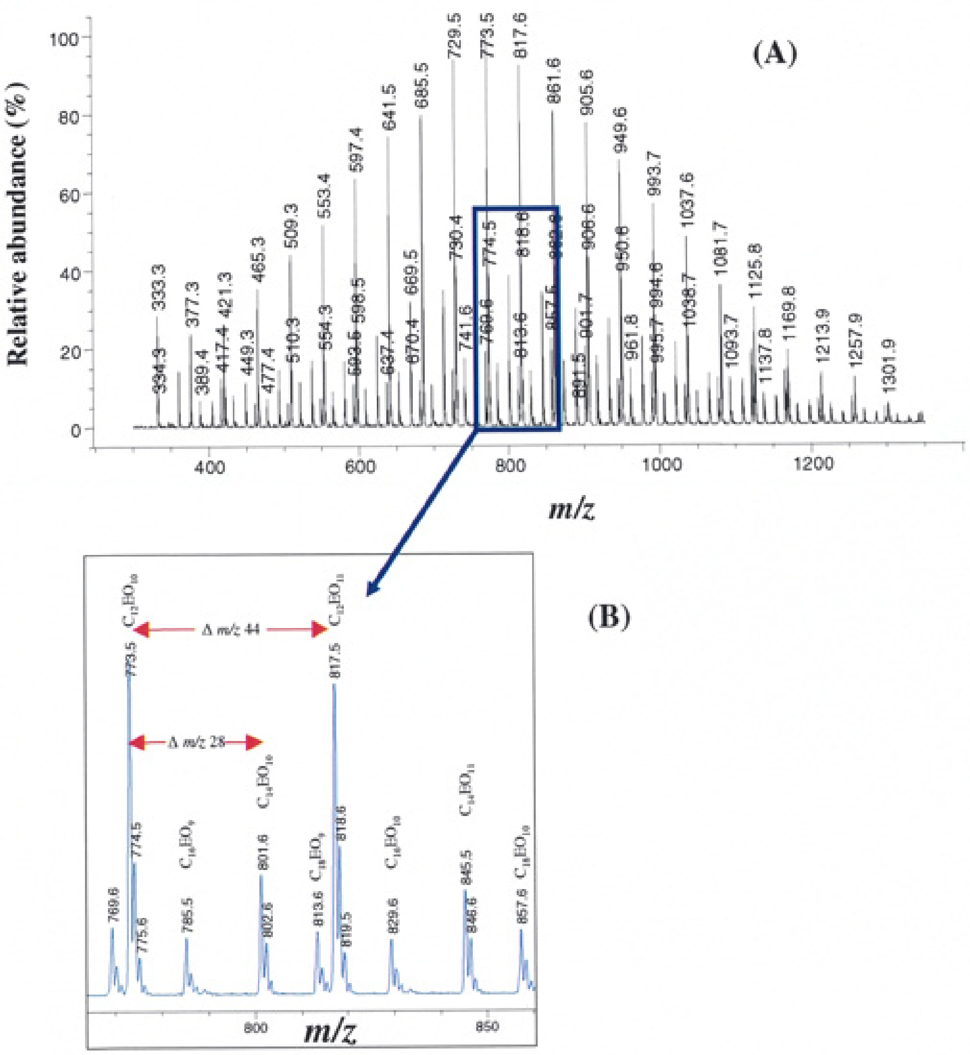
Conditions- ESI-MS with gas temperature 300 °C, gas flow 7 L/min, nebuliser 50 psi and capillary voltage 4000 V for (A) positive ion (B) negative ion, of approximately 0.4 mg/mL solutions in mobile phase (water/ACN/THF/ammonia, 500/450/50/1, v/v) using syringe infusion at 200 μL/min.



Preparation and dilution of derivatives for Genapol C100 and Genapol T100 allowed mass spectral data to be collected in negative ESI mode as shown in Figure 6.6 and 6.7. The magnified region in Figure 6.6 (B) clearly shows that although Genapol C100 is predominantly a mixture of C_{12} and C_{14} AEs, there are also C_{16} and C_{18} AEs present.

Figure 6.6. Negative ion ESI-MS of a Genapol C100 derivative

Conditions- negative ion ESI-MS with gas temperature 300 °C, gas flow 7 L/min, nebuliser 50 psi and capillary voltage 4000 V, of an approximately 0.4 mg/mL solution in mobile phase (water/ACN/THF/ammonia, 500/450/50/1, v/v) using syringe infusion at 50 μL/min.



The magnified region (Figure 6.6 (B)) highlights characteristic Δ *m/z* values seen for the spectrum of AE, with Δ *m/z* 28 indicating a difference of 2 carbon units (C_2H_4) in the alkyl chain and Δ *m/z* 44 indicating different degrees of ethoxylation. The data is consistent with that supplied by Clariant for Genapol C100 [7] as shown in Table 6.2. The percentages of each homologue present are in the correct order of magnitude in the spectrum presented in Figure 6.6 (A). By comparing ions close to the 100 % normalised signal for $C_{12}EO_{10}$ (*m/z* 773.5), using the scale on the y-axis, the following approximate ratios can be deduced:

$C_{12}EO_{10} : C_{14}EO_{10} : C_{16}EO_9 : C_{18}EO_9$ = 100: 40:15:15
Which on normalisation to 100 % gives:
$C_{12}EO_{10} : C_{14}EO_{10} : C_{16}EO_9 : C_{18}EO_9$ = 58: 23: 9: 9

Although only providing an estimate of the true homologue distribution, the data above compares well with that supplied by the manufacturer (Table 6.2). The average EO number can also be estimated to be around 10 from this maximum signal which agrees with NMR data (Section 6.10).

Table 6.2 Distribution of AEs in Genapol C100 and Genapol T110 as supplied by Clariant [7]

ALCOHOL ETHOXYLATE	**% C_{12}**	**% C_{14}**	**% C_{16}**	**% C_{18}**	**% C_{20}**
Genapol C100	48 – 58	19 – 24	9 –12	11 - 14	
Genapol T110		Max 7	25 – 35	60 - 75	Max 6

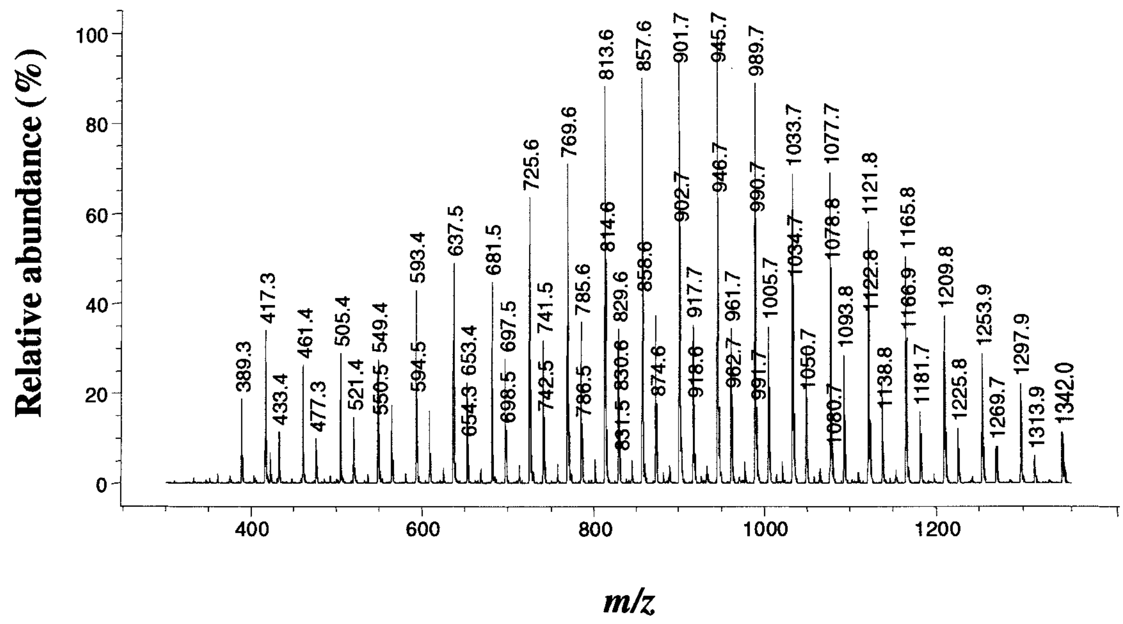
Similarly for Genapol T110 the spectrum in Figure 6.7 shows this to be predominantly a mixture of C_{18} and C_{16} AEs, again being ionised to give the homologue distribution consistent with that shown in Table 6.2. If we consider the 100 % normalised signal for $C_{18}EO_{12}$ at *m*/z 945.7 and compare this to the signal for $C_{16}EO_{13}$ at *m/z* 961.7 (35 %), then there is approximately 75 % C_{18} and 25 % C_{16} in Genapol T110. These figures will be slightly lower than this if we account for other homologues present in this sample, which can be estimated at around 5 % from Figure 6.7., seen as the smaller signals equally spaced in between the major ions.

The average EO number for Genapol T110 can also be estimated to be around 12 from this maximum signal which agrees with NMR data (Section 6.10).

The commercial samples appear to give the correct homologue and EO distribution when analysed as phthalic anhydride derivatives, both in comparison to manufacturer information and characterisation analysis carried out by another laboratory (Section 6.10). Indeed the homologue distribution for the Genapols should also reflect the oleochemical starting materials, Genapol C100 and T110 are coconut and tallow derived, respectively.

Figure 6.7. Negative ion ESI-MS of a Genapol T110 derivative

Conditions- negative ion ESI-MS with gas temperature 300 °C, gas flow 7 L/min, nebuliser 50 psi and capillary voltage 4000 V, of an approximately 0.4 mg/mL solution in mobile phase (water/ACN/THF/ammonia, 500/450/50/1, v/v) using syringe infusion at 50 μL/min.



6.6 *Derivatisation efficiency*

At this stage it was prudent to check derivatisation efficiency. An efficient derivatisation would provide the basis of a stable and reproducible derivative for use in quantifying AEs in environmental samples. The method was now working well in negative ionisation ESI, however any underivatised material would not be ionised in this mode of analysis. Therefore this experiment reverted back to positive ionisation that would be able to simultaneously analyse for both the derivative and any remaining underivatised material for selected AEs.

1 mL of pyridine containing 4 mM each of $C_{12}EO_0$, $C_{12}EO_6$, $C_{12}EO_{12}$ and $C_{12}EO_{20}$ was derivatised with 60 mg (400 mM) of phthalic anhydride at 85 °C for 1 hr. This equates to a 25 times molar excess of derivatisation reagent to AE. An aliquot of the same solution was left underivatised. Both solutions were diluted 100-fold in mobile phase. By using syringe infusion an area comparison of AE remaining underivatised in the derivatised sample to area of the same component in the underivatised sample was made. The results are displayed in Table 6.3.

Table 6.3. Derivatisation efficiency- calculated by comparing the syringe infusion of EOs in derivatised and underivatised form

Conditions- positive ion ESI-MS with gas temperature 300 °C, gas flow 7 L/min, nebuliser 40 psi and capillary voltage 4000 V for 40 μM solutions in mobile phase (water/ACN/THF/ammonia, 500/450/50/1, v/v) using syringe infusion at 50 μL/min.

AE	**Ion $[M + NH_4]^+$ (*m/z*)**	**Area in underivatised sample**	**Area in derivatised sample**	**% remaining underivatised**
$C_{12}EO_0$	204.2	nd	nd	nd
$C_{12}EO_6$	468.4	101408	967	0.95
$C_{12}EO_{12}$	732.6	397888	4790	1.2
$C_{12}EO_{20}$	1084.8	83896	1956	2.3

nd = not detected

The data in Table 6.3 demonstrates a derivatisation efficiency of > 97% for the AE compounds analysed.

6.7 Preliminary LC method development (miniaturisation of LC)

The method was originally set up on a Hypersil BDS C_8 4.6 mm inner diameter (i.d.) x 25 cm (5 μm) column with a flow rate of 1 mL/min. The gradient was as shown in Table 6.4 where mobile phase A and B were as listed in Section 5.5.

Table 6.4 Original gradient of 4.6 mm i.d. column

Time (min)	Mobile phase A	Mobile Phase B
0	65	35
8	65	35
22.5	50	50
30.0	0	100
40.0	0	100

(Re-equilibration at Time 0 conditions was 10 min)

3 μg of a total AE derivative were injected onto the column with the MS operated at a gas temperature of 300 °C, flow 7 L/min, nebuliser pressure 50 psi and a fragmentor ramp as listed in Section 5.5. This approach gave good peak shape of the extracted ions and signal to noise values as displayed in Figure 6.8 (A).

One aim in method development was to scale down the separation onto a narrower i.d. column e.g. 2 mm. The reason behind this approach was because the flow rate of 1 mL/min however was not optimum for the pneumatically assisted ESI, which was known to give optimum sensitivity in the region of 300 μL/min [8]. In addition to this, column theory [9] also suggests that a narrower id column will also give an increase in sensitivity for the same volume injected according to the equation:

$$C_{max} = \frac{4M\sqrt{N}}{\pi d^2 \varepsilon L(1+k')\sqrt{2\pi}} \qquad [6.1]$$

Where M is mass injected, N is plate number, d is column diameter, L is column length, k' is capacity ratio and ε is porosity.

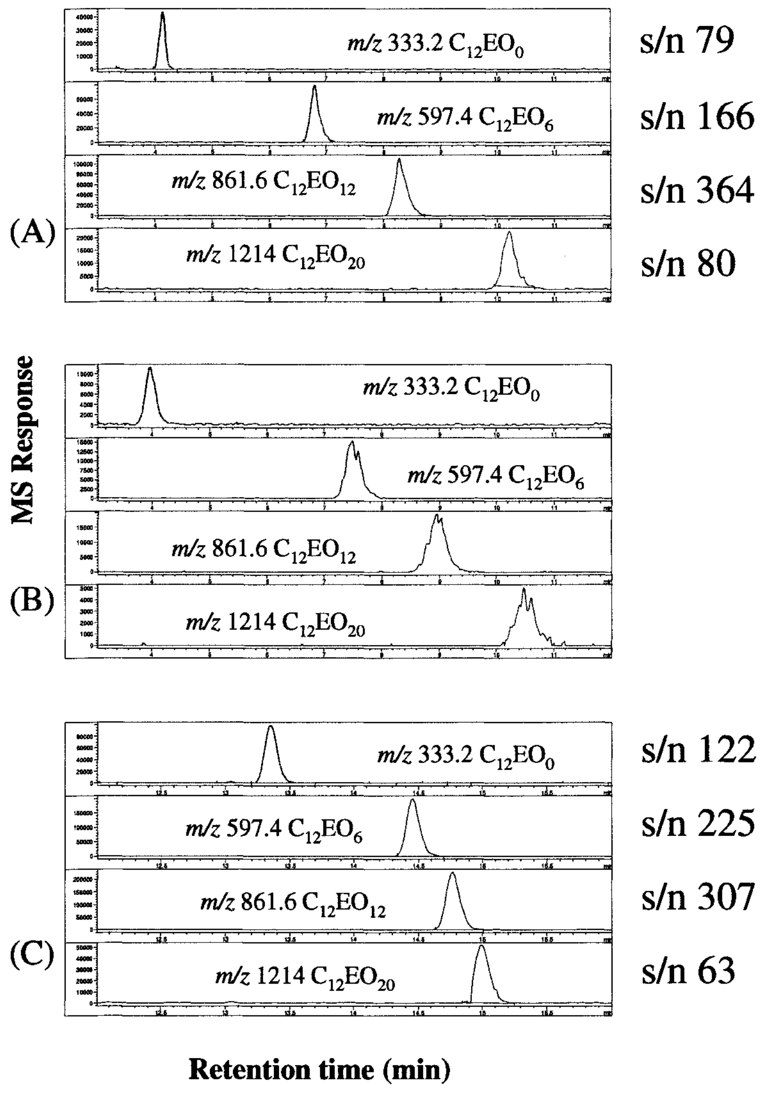
It follows from equation [6.1] that when injecting the same absolute mass on two columns with all other key parameters, such as plate numbers and length being similar, but different inner diameters, a higher concentration is obtained for the column with a smaller inner diameter. However it should be remembered that the 4.6 mm i.d. column is actually capable of having more sample loaded onto it and the 1mL/min flow could be split to introduce 200 μL/min into the MS. Therefore the absolute detection limit will only be improved by using a narrower id column in situations where the amount of sample is limited.

A narrow bore column (Luna C_8 2.0 mm i.d. column (15 cm, 5 μm)) was chosen to scale down the flow rate and examine the theory presented. The high carbon loading in this type of column was reported to protect the silica from dissolving in high pH mobile phase, up to pH 10, which would be beneficial in the conditions used in this method. Initial attempts however at reproducing the separation with conditions as close as possible to the 4.6 mm column (a flow of 0.25 mL/min was used and the nebuliser was dropped to 35 psi), gave extracted ions with a very poor peak shape as shown in Figure 6.8. The reason for this was not entirely clear but altering the initial mobile phase composition to contain a higher aqueous content solved the problem (conditions as Section 5.5). Using this adjustment to the method and the 2.0 mm i.d. column the following chromatography and extracted ions were obtained with signal to noise values as displayed in Figure 6.8 (C).

The sensitivity obtained was comparable for the two columns. The theoretical increase in sensitivity was not realised, but on reflection the scaling down experiment should have been carried out on identical columns i.e. same length, manufacturer but differing internal diameter only. The 4.6 mm i.d. column was also run using the higher aqueous initial mobile phase composition but it was found that at 1 mL/min the source was not as stable at the temperature and gas flows used. This is a common effect with the highly aqueous mobile phase at the start of the run leading to the possibility of electrical shorting within the ion source. The other advantage of the lower flow rate on the 2.0 mm i.d. column is the decreased use of solvents which as well as the reduced cost means that larger sequences of samples can be analysed without the need to replenish solvents. The new gradient also allowed a divert time of 8 min (flow goes to waste and not to the MS) to be used to prevent source contamination with excess derivatisation agent, but at the same time allowing stabilisation of the MS before elution of the first analyte at approximately 13 min.

Figure 6.8. Comparison of extracted ions:
(A) Hypersil BDS (5 µm) 25 cm x 4.6 mm i.d., flow 1 mL/min
(B) Luna C_8 (5 µm) 15 cm x 2.0 mm i.d., flow 0.25 mL/min (gradient as A))
(C) Luna C_8 (5 µm) 15 cm x 2.0 mm i.d., flow 0.25 mL/min (modified gradient)
(including a signal to noise (s/n) comparison of columns in (A) and (C))

Conditions- 3 µg of total AE derivative injected onto the column, ESI-MS with gas temperature 300 °C, gas flow 7 L/min, nebuliser 50 psi for (A), 35 psi for (B) and (C), and capillary voltage 4000 V. For gradient details see text.



At this stage however the LC/MS method developed on the Luna C_8 (5 μm) 2.0 mm i.d. column looked fit for purpose and provided a good solution to link the LC with the MS with the pneumatically assisted electrospray. The method would be more rigorously tested in future work and the ultimate test would be on the influence of extracts from environmental samples.

6.8. LC method development (as applied to commercial samples)

A calibration over the range of 0, 7.5, 15, 30 and 60 μg/mL total AE was constructed for each of the ions listed in Table 6.5. The correlation coefficients for these curves are listed in Table 6.6. Good results were obtained with correlation coefficients principally >0.99 obtained for the majority of the ethoxylate species. The interference noted in Table 6.6 was an isobaric interference at *m/z* 845.6 for $C_{14}EO_{11}$. The method was equally well suited to Luna C_{18} or C_{18} (2) phases. Typical total ion chromatograms (TICs) of a standard mixture of the commercial samples and extracted ion chromatograms (EICs) separated on a C_{18} (2) phase are shown in Figure 6.9. Extracted ion chromatograms for selected AE ethoxymers for Genapol C100 and Lutensol A07 are shown in Figure 6.9 ((B) and (C), respectively).

Although roughly separated into alkyl homologues, note the overlap of extracted ions within the C_{12} and C_{13} windows. There is an increase in retention for increasing EO groups. This is not consistent with the separation of unaltered AE with acetonitrile, THF and water mobile phases [10] where retention time was generally found to be inversely proportional to the ethoxylation degree. However the opposite effect was observed for 1- naphthyl isocyanate derivatives [10]. The latter observation is consistent with the phthalate derivatives in this work. The observed spread in retention is probably beneficial in terms of ionisation in the MS, as co-eluting peaks would compete against each other in the ionisation process.

Lutensol A07 is an "oxo" AE which contains β-methyl and β-ethyl isomers as well as linear species, whilst Genapol C100 is linear with no branched isomers. The EIC trace for Genapol C100 AE is shown in Figure 6.9 (B), whilst that for Lutensol A07

(Figure 6.9 (C)) clearly shows the resolution of branched and linear AE isomers, for EO_0 and EO_1, under the HPLC conditions employed.

Table 6.5 Negative ions *(m/z)* used to generate extracted ion chromatograms for standards and spiked samples. The ions listed are $[M-H]^-$ for the derivatised AE species.

	IONS (*m/z*)					
	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	333.2	347.2	361.3	375.3	389.3	417.3
EO_1	377.3	391.3	405.3	419.3	433.3	461.4
EO_2	421.3	435.3	449.3	463.3	477.4	505.4
EO_3	465.3	479.3	493.4	507.4	521.4	549.4
EO_4	509.4	523.4	537.4	551.4	565.4	593.4
EO_5	553.4	567.4	581.4	595.4	609.4	637.5
EO_6	597.4	611.4	625.4	639.5	653.5	681.5
EO_7	641.4	655.5	669.5	683.5	697.5	725.5
EO_8	685.5	699.5	713.5	727.5	741.5	769.6
EO_9	729.5	743.5	757.5	771.6	785.6	813.6
EO_{10}	773.5	787.6	801.6	815.6	829.6	857.6
EO_{11}	817.6	831.6	845.6	859.6	873.6	901.7
EO_{12}	861.6	875.6	889.6	903.6	917.7	945.7
EO_{13}	905.6	919.6	933.7	947.7	961.7	989.7
EO_{14}	949.7	963.7	977.7	991.7	1005.7	1033.8
EO_{15}	993.7	1007.7	1021.7	1035.7	1049.8	1077.8
EO_{16}	1037.7	1051.7	1065.8	1079.8	1093.8	1121.8
EO_{17}	1081.8	1095.8	1109.8	1123.8	1137.8	1165.9
EO_{18}	1125.8	1139.8	1153.8	1167.8	1181.9	1209.9
EO_{19}	1169.8	1183.8	1197.8	1211.9	1225.9	1253.9
EO_{20}	1213.8	1227.9	1241.9	1255.9	1269.9	1297.9

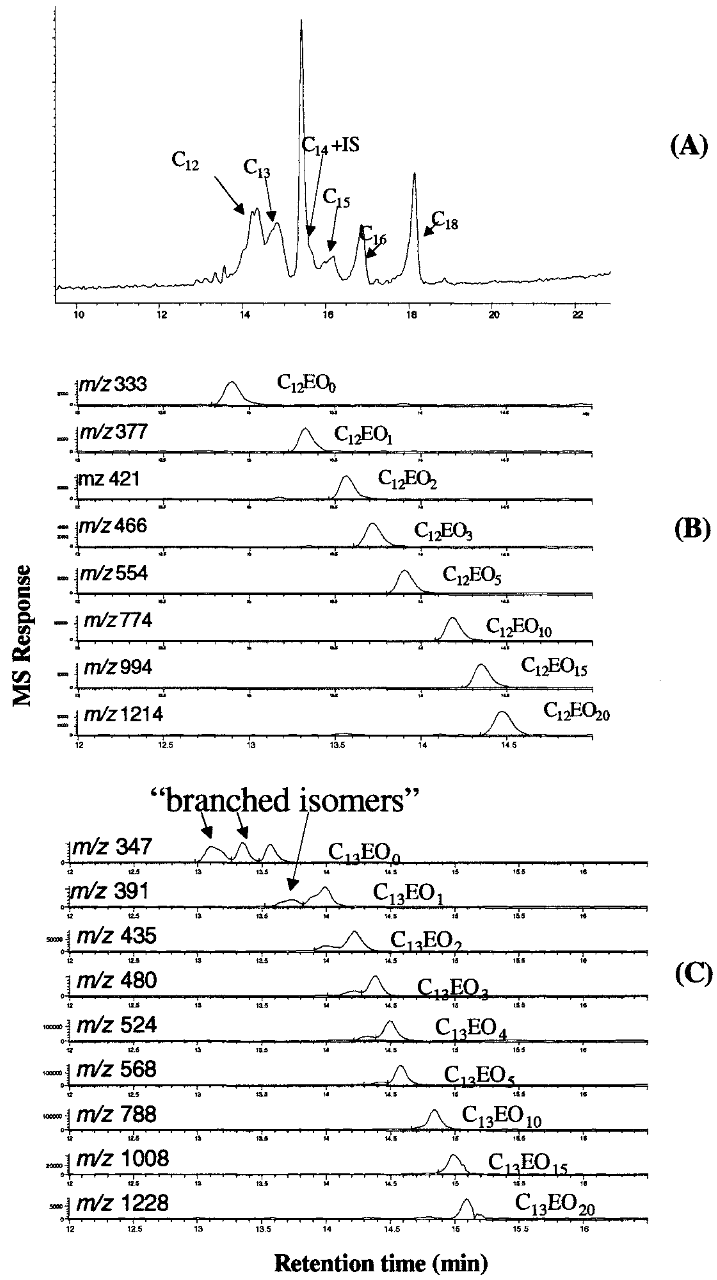
Table 6.6. Correlation coefficients for extracted ion data over the concentration range 0-60 μg/mL total AE (n = 5).

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	0.9992	0.9990	0.9952	0.9951	0.9956	0.9959
EO_1	0.9894	0.9989	0.9995	0.9740	0.9943	0.9987
EO_2	0.9994	0.9959	0.9957	0.9902	0.9943	0.9979
EO_3	0.9972	0.9937	0.9917	0.9929	0.9984	0.9993
EO_4	0.9983	0.9945	0.9971	0.9983	0.9975	0.9995
EO_5	0.9969	0.9725	0.9975	0.9970	0.9989	0.9962
EO_6	0.9901	0.9948	0.9961	0.9936	0.9984	0.9965
EO_7	0.9953	0.9964	0.9973	0.9733	0.9943	0.9966
EO_8	0.9972	0.9961	0.9968	0.9977	0.9960	0.9929
EO_9	0.9954	0.9928	0.9954	0.9963	0.9928	0.9921
EO_{10}	0.9944	0.9943	0.9990	0.9949	0.9984	0.9916
EO_{11}	0.9912	0.9933	Int	0.9973	0.9962	0.9889
EO_{12}	0.9942	0.9968	0.9983	0.9973	0.9867	0.9821
EO_{13}	0.9960	0.9949	0.9932	0.9983	0.9912	0.9835
EO_{14}	0.9981	0.9988	0.9928	0.9955	0.9902	0.9870
EO_{15}	0.9909	0.9909	0.9993	0.9925	0.9978	0.9880
EO_{16}	0.9914	0.9911	0.9896	0.9812	0.9952	0.9913
EO_{17}	0.9966	0.9923	0.9658	0.9873	0.9966	0.9996
EO_{18}	0.9973	0.9993	0.9876	0.9996	0.9833	0.9794
EO_{19}	0.9978	0.9480	0.9939	0.9536	0.9904	0.9999
EO_{20}	0.9679	0.9931	0.9983	0.9232	0.9917	0.9802

Int = isobaric interference

Figure 6.9. LC/MS Chromatograms for AE from a standard mixture containing equal amounts of Lutensol A07, Genapol C100 and Genapol T110. (A) Total ion chromatogram at a concentration of 60 µg/mL. (B) Extracted ion chromatograms for Genapol C100 showing selected C_{12}AE species. (C) Extracted ion chromatograms for Lutensol A07 showing selected C_{13}AE species.

Conditions- Luna C_{18} (2) 150 x 2.0 mm i.d. 5 µm, flow 0.25 mL/min, mobile phase A, 0.1 % v/v ammonia in Ultrapure water and B, 0.1 % v/v ammonia in 900/100 (v/v) ACN/THF. Gradient 15 % to 100 % B in 25 min, 10 µL injection.



6.9 *LC method development: LOQ determination*

Determination of the instrumental limit of quantification (LOQ) was carried out using the mixture of individual EOs (section 5.1.4). These were prepared as a 100 μM mixed derivative and serially diluted in derivatisation reagent to give 10 μM, 1 μM, 100 nM, 10 nM and 1 nM concentrations. These solutions were then analysed by LC/MS (Section 5.5.1) to provide an assessment of LOQ. On inspection of the extracted ion data for the 10 nM solution, signals were obtained that were that were not significantly greater than the noise in the chromatogram. An evaluation of LOQ was made from extracted ions in the 100 nM AE derivative (signal to noise 10 to 1). The signal to noise values presented in Table 6.6, were calculated using the Agilent Chemstation software. An LOQ value was calculated from the concentration of each EO in the mix and extrapolated to a signal to noise value of 10 to 1 (Table 6.6).

Figure 6.10 Extracted ion chromatograms of EOs injected from a 100 nM solution

Conditions- Luna C_{18} (2) 150 x 2.0 mm i.d. 5 μm, flow 0.25 mL/min, mobile phase A, 0.1 % v/v ammonia in Ultrapure water and B, 0.1 % v/v ammonia in 900/100 (v/v) ACN/THF. Gradient 15 % to 100 % B in 25 min, 10 μL injection.

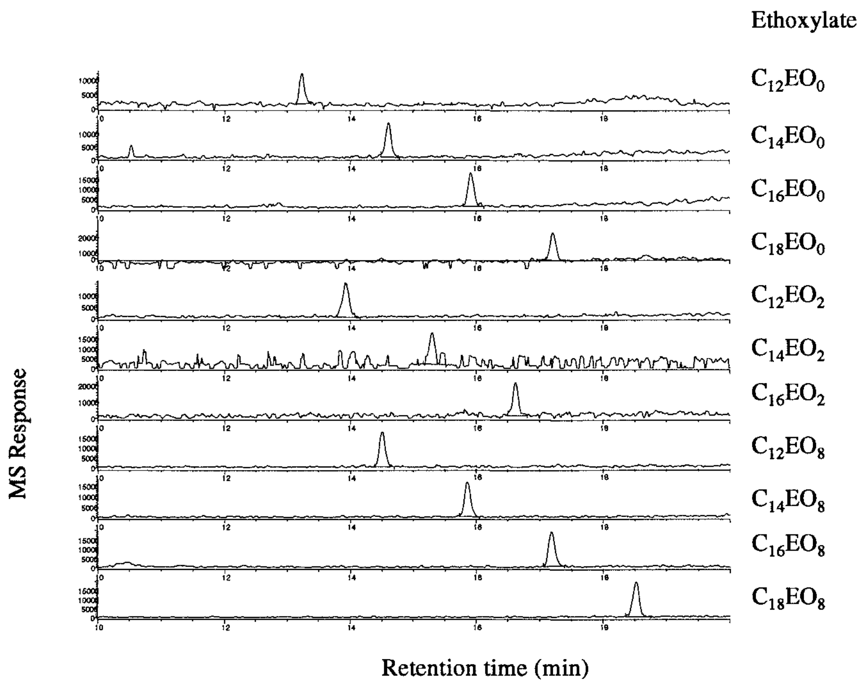


Table 6.6. LOQ of individual EOs (signal to noise 10 to 1)

ETHOXYLATE	MW	CONC (ng/mL) IN 100 nM MIX	AREA	PEAK TO PEAK S/N	LOQ (ng/mL) 10/1 S/N
C12EO0	186.3	18.63	63,940	8	23
C14EO0	214.4	21.44	86,084	10	21
C16EO0	242.4	24.24	112,213	16	15
C18EO0	270.5	27.05	122,322	12	23
C12EO2	274.4	27.44	117,197	11	25
C14EO2	302.5	30.25	84,308	3	101
C16EO2	330.5	33.05	128,547	6	55
C12EO8	538.8	53.88	113,677	12	45
C14EO8	566.8	56.68	117,860	21	27
C16EO8	594.9	59.49	133,233	22	27
C18EO8	622.9	62.29	134,348	21	30
Mean LOQ					**36**

The data in Table 6.6 demonstrates a mean LOQ of approximately 40 ng/mL (400 pg injected on column) for the ethoxylates tested. It also shows a very balanced molar response of the different EOs, by reference to the peak areas obtained.

6.10 Characterisation of commercial samples

6.10.1 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

Experimental

NMR analysis (^{1}H and ^{13}C at 600 and 150 MHz, respectively) was conducted to confirm the extent of the alkyl chain branching characteristics and the average EO length of the three nonionic samples [11]. All samples were subjected to derivatisation [12] by dissolving a few mg of the product in deuterated chloroform (99.5% deuteration) obtained from Merck (Darmstadt, Germany) and subsequent addition of a few droplets of trichloroacetylisocyanate obtained from Aldrich (Milwaukee, WI). All NMR experiments were performed at 303 K, on a Bruker DMX600 spectrometer. Samples for ^{13}C NMR measurements were put in 10 mm tubes, and a 30 sec relaxation delay was used (in order to prevent signal saturation). The ^{1}H NMR spectra were recorded in 5 mm tubes, with a relaxation decay of 15 sec. The NMR datasets were processed with the Bruker XWINMR software, operating on Unix workstations. Quantitative data on branching characteristics and average EO length were obtained by integration of assigned regions in the ^{13}C and ^{1}H NMR spectra, respectively. Table 6.7 shows the regions integrated in the ^{1}H NMR spectra, with the calculation for average EO number shown in Equation [6.2].

Results and discussion

The NMR method for determination of average EO number relies on the integration of identified signals (Table 6.7) in the NMR spectrum. From an NMR point of view there are two types of compounds present in AEs: R-(EO)n-OH and R-OH. (the alkyl chains R can also be branched). Determination of the average EO-number relies on the shift of the CH_2-group next to the alcohol group to lower field after esterification of that group, so that the end-CH_2-groups can be clearly distinguished. Esterification of the hydroxyl groups can be performed in a straightforward manner by the addition of a reactive isocyanate. This method is simple, rapid and gives a large downfield shift of the CH_2 resonance adjacent to the esterified hydroxyl groups.

Table 6.8 shows branching as determined by ^{1}H and ^{13}C NMR and average EO number as calculated from the ^{1}H NMR data. The purity of the commercial samples (5.1.2) was also determined to be > 98 % from NMR.

6.10.2 MASS SPECTROMETRY

Experimental

65 mg of sample were dissolved in 3 mL of dichloromethane. To this 95 mg of 2-fluoro-N-methylpyridinium p-toluenesulphonate obtained from Sigma (Milwaukee, WI) were added. The mixture was stirred on a magnetic stirrer for 30 minutes and was typically cloudy in appearance. At this point 52 μL of triethylamine (Sigma) were added, with stirring continued for another 30 minutes until the mixture became clear. A volume of 5 mL of the reaction mixture was dissolved in 20 mL of methanol and measured using syringe infusion with positive ion ESI-MS on an Agilent 1100 LC/MSD.

Results and discussion

MS was used to determine EO and alkyl chain distribution and results from the syringe infusion ESI-MS are shown in Table 6.9. The values in Table 6.10 are % w/w values of individual EO in a combined standard prepared with equal weights of Lutensol A07, Genapol C100 and Genapol T110, as used in Section 5.1.3. The C-chain distribution in the mixed standard was C_{12} (19.91), C_{13} (23.34), C_{14} (7.04), C_{15} (9.99), C_{16} (14.30) and C_{18} (25.42) % w/w.

Table 6.7 Identified and assigned spin systems of Lutensol A07

NONIONIC	IDENTIFIED AND ASSIGNED $\mathbf{CH_2}$-GROUPS	CHEMICAL SHIFT (PPM)	INTEGRAL
Linear 0	R-$\mathbf{CH_2}$-O-TCAI	4.2	(L_0)
Linear n	R-O(CH_2-CH_2O)$_n$-$CH_2$$\mathbf{CH_2}$O-TCAI	4.4	(L_n)
Linear n	R-$\mathbf{CH_2}$-O($\mathbf{CH_2}$-$\mathbf{CH_2}$O)$_n$-$\mathbf{CH_2}$$CH_2$O-TCAI	3.8 – 3.4	(EO_n)
Branched 0	R-CH(CH_3)-$\mathbf{CH_2}$O-TCAI	4.0 – 4.15	($B_0$1)
Branched 0	R'-CH(R")-$\mathbf{CH_2}$O-TCAI	4.15	($B_0$2)
Branched n	R-CH(CH_3)-$\mathbf{CH_2}$-O(CH_2-CH_2O)$_n$-$CH_2$$CH_2$O-TCAI	3.3 – 3.15	(EO_n)
Branched n	R'-CH(R")-$\mathbf{CH_2}$-O(CH_2-CH_2O)$_n$-$CH_2$$CH_2$O-TCAI	3.3	(EO_n)

Calculation

$$\textit{Average EO-number} = \frac{\textit{number of EO groups}}{\textit{total number of chains}} = \frac{(\sum(EO_n))/4}{((L_n)+(L_0)+(B_0 1)+B_0 2))/2} \qquad [6.2]$$

Table 6.8 Summary of NMR data

Conditions as described in text (data provided by Unilever Research Vlaardingen)

		Lutensol A07		Genapol C100		Genapol T110	
		Ethoxylate	Alcohol	Ethoxylate	Alcohol	Ethoxylate	Alcohol
^{1}H NMR	H	56.1	3.6	94.3	5.7	96.2	3.8
	Methyl	17.9	3.6	0	0	0	0
	≥Ethyl	15.2	3.6	0	0	0	0
	EO number	6.8		9.8		12.1	
		Ethoxylate	Alcohol	Ethoxylate	Alcohol	Ethoxylate	Alcohol
^{13}C NMR	H	55	4	100	0	100	0
	Methyl	20	0	0	0	0	0
	Ethyl	7	0	0	0	0	0
	≥Propyl	14	0	0	0	0	0

Table 6.9 % w/w distribution of Lutensol A07, Genapol C100 and Genapol T110 as determined by MS

Conditions- Syringe infusion using an Agilent 1100 LC/MS with positive ionisation ESI-MS of pyridinium derivatives (data provided by Unilever Research Vlaardingen)

	Lutensol A07		Genapol C100				Genapol T110	
	C_{13}	C_{15}	C_{12}	C_{14}	C_{16}	C_{18}	C_{16}	C_{18}
EO_0	3.94	1.96	1.59	0.64	0.09	0.26	0.31	2.37
EO_1	3.02	1.33	1.14	0.49	0.19	0.40	0.77	1.33
EO_2	3.27	1.55	1.16	0.52	0.25	0.61	0.83	1.18
EO_3	4.20	1.90	1.76	0.54	0.26	0.51	0.98	1.36
EO_4	5.51	2.66	2.33	0.98	0.24	0.47	1.19	2.45
EO_5	6.48	2.85	2.60	1.18	0.53	0.61	1.31	2.79
EO_6	6.29	2.85	3.35	1.04	0.61	0.71	1.52	3.03
EO_7	6.36	2.64	3.91	1.41	0.71	0.69	2.12	3.49
EO_8	6.17	2.63	4.63	1.71	0.92	0.81	2.27	4.49
EO_9	5.54	2.32	5.14	1.81	0.88	0.83	2.64	4.78
EO_{10}	5.23	2.08	5.17	2.01	0.83	0.82	2.72	5.20
EO_{11}	4.45	1.52	5.38	1.81	0.85	0.74	3.05	5.32
EO_{12}	3.41	1.08	4.96	1.73	0.78	0.61	2.41	6.03
EO_{13}	2.48	0.95	3.84	1.34	0.57	0.66	2.68	5.53
EO_{14}	1.19	0.73	3.53	1.36	0.52	0.53	2.23	3.90
EO_{15}	1.29	0.41	3.07	0.80	0.60	0.40	1.82	3.97
EO_{16}	0.58	0.25	2.17	0.68	0.27	0.21	1.63	2.53
EO_{17}	0.08	0.17	1.38	0.54	0.18	nd	1.28	2.18
EO_{18}	0.26	0.09	1.10	0.35	nd	nd	0.86	1.60
EO_{19}	0.18	0.00	0.66	0.18	nd	nd	0.70	1.13
EO_{20}	0.09	0.00	0.44	nd	nd	nd	0.29	0.93
EO_{21}	nd	nd	0.19	nd	nd	nd	nd	0.44
EO_{22}	nd	nd	0.20	nd	nd	nd	nd	0.34
Total	70.02	29.98	59.72	21.12	9.29	9.87	33.61	66.39

nd = not detected

Table 6.10 % w/w values in an AE standard containing an equal weight of Lutensol A07, Genapol C100 and Genapol T110

Calculated from data provided in Table 6.9.

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	0.53	1.31	0.21	0.65	0.13	0.88
EO_1	0.38	1.01	0.16	0.44	0.32	0.58
EO_2	0.39	1.09	0.17	0.52	0.36	0.60
EO_3	0.59	1.40	0.18	0.63	0.41	0.62
EO_4	0.78	1.84	0.33	0.89	0.48	0.97
EO_5	0.87	2.16	0.39	0.95	0.62	1.13
EO_6	1.12	2.10	0.35	0.95	0.71	1.25
EO_7	1.30	2.12	0.47	0.88	0.94	1.40
EO_8	1.54	2.06	0.57	0.88	1.06	1.77
EO_9	1.71	1.85	0.60	0.77	1.18	1.87
EO_{10}	1.72	1.74	0.67	0.69	1.18	2.01
EO_{11}	1.79	1.48	0.60	0.51	1.30	2.02
EO_{12}	1.65	1.14	0.58	0.36	1.06	2.21
EO_{13}	1.28	0.83	0.45	0.32	1.08	2.06
EO_{14}	1.18	0.40	0.45	0.24	0.92	1.48
EO_{15}	1.02	0.43	0.27	0.14	0.81	1.46
EO_{16}	0.72	0.19	0.23	0.08	0.63	0.91
EO_{17}	0.46	0.03	0.18	0.06	0.48	0.73
EO_{18}	0.37	0.09	0.12	0.03	0.29	0.53
EO_{19}	0.22	0.06	0.06	nd	0.23	0.38
EO_{20}	0.15	0.03	nd	nd	0.10	0.31
EO_{21}	0.06	nd	nd	nd	nd	0.15
EO_{22}	0.07	nd	nd	nd	nd	0.11
Total C-chain	19.91	23.34	7.04	9.99	14.30	25.42

nd = not detected

6.11 Conclusion

In this chapter the development of an LC/ESI-MS method for the analysis of $C_{12\text{-}18}EO_{0\text{-}20}$ AEs utilising negative mode ionisation of phthalic anhydride derivatives is described. The benefit of derivatisation and LC/MS, giving a more molar response of AEs and a fingerprint consistent with NMR data is demonstrated. The LC method was miniaturised to a narrow bore column and a mean LOQ for the derivatives was approximately 40 ng/mL (400 pg injected on column). The method developed was used to analyse extracts of AEs in environmental samples in Chapter 7 and 8, respectively.

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EVALUATION AND APPLICATION OF SPE AND LC/MS TO SEWAGE INFLUENT AND EFFLUENT SAMPLES

7.1 Introduction

The liquid chromatography/mass spectrometry (LC/MS) method developed in Chapter 6 has shown satisfactory performance in the analysis of alcohol ethoxylate (AE) standards. In the next stage of method development AEs spiked at realistic environmental levels in environmental waters will be analysed to examine the effect of a concentration step and resulting co-extracted material (matrix effects). The concentration step for these analytes is best carried out by solid phase extraction (SPE) (see Chapter 3). For AE extraction from aqueous samples, well documented procedures exist utilising reversed-phase SPE cartridges e.g. C_8 [1] and C_{18} [2]. A C_2 phase was used by Dunphy et al. [3], which they stated retained the wide range of polarity of analytes present in commercial AEs better than C_8 or C_{18} Preliminary work in this study was using a C_2 phase in combination with a simplified elution system (methanol only) to examine recovery of AEs, any shortfalls could be addressed later by addition of other solvents of the appropriate polarity.

7.2 Preliminary work

Preliminary method development was carried out using Lutensol A07. A standard was prepared by derivatising 5 mg of Lutensol A07 in 5 mL of derivatisation reagent (Section 5.1.3). An equivalent amount of Lutensol A07 was also spiked into 500 mL each of Ultrapure water, synthetic river water (Elendt [4]) and stream water (Sharn Brook, Sharnbrook, Bedfordshire) before taking through the SPE procedure as follows: C_2 500 mg/6 mL Isolute cartridges from Argonaut (Hengoed, UK) were conditioned with two cartridge volumes of methanol and water. The samples were loaded from a 500 mL bottle using a Vac Elut manifold from Varian (Walton-on-Thames, UK), polypropylene tubing, adaptors and vacuum pump at a flow rate of

5-10 mL/min (Figure 3.4). The cartridges were dried under vacuum for at least 1 hr before eluting with 10 mL of methanol. The extracts were evaporated and derivatised (as in Section 5.4., but no internal standard)

Analysis by LC/MS was carried out as in Section 5.5.1. Extracted ion area comparisons of the SPE recovered samples for $C_{13}EO_{0-20}$ and $C_{15}EO_{0-20}$ with those of the directly derivatised standard are shown in Table 7.1. The 'drying only' data refers to 5 mg of Lutensol A07 spiked into 10 mL of methanol and concentrated to dryness under nitrogen before derivatising. Visually the data in Table 7.1 has been presented to show possible lower recovery of AE for the EO_{0-4} species, whereas the recoveries for EO_{5-20} are generally higher. For example the stream sample has mean recoveries of $C_{15}EO_{0-4}$ of 68 % and $C_{15}EO_{5-20}$ of 95 %.

The means of $C_{15}EO_{0-4}$ AE recovery for the different media types are compared statistically in Table 7.2. The calculations presented were performed using the "T-test: two-sample assuming equal variances" formula in Excel™. Column two of this table compares the mean of drying only to SPE recovery from Ultrapure water. The calculated result for t Stat (7.9) > t Critical two-tail (2.3), suggests that there is a 95 % probability that the means are significantly different. Similar comparisons made in columns three and four of the table show that the means of drying only compared to stream recovery and also Ultrapure water compared to Stream recovery are also significantly different. This data supports the initial visual assessment and shows there may be a problem in recovery of some of the lower EOs.

However there appeared to be minimal losses of the more volatile AEs on the evaporation step and the transfer of the methodology to the Autotrace, important in both automation as well as allowing more consistent load flows and more efficient cartridge drying with nitrogen, was comparable to the Vac Elut method. The preliminary work was done at high concentrations of Lutensol A07 and in relatively clean media. The next stage of method development was to look at lower levels in a sample containing a higher organic content such as a final effluent

Table 7.1. Percent recovery data for Lutensol A07 spiked at 5 mg/ 500 mL in different media on a C_2 500 mg Isolute cartridge followed by elution with 10 mL of methanol. Results quoted as mean % (individual replicates), n = 2

	STREAM		ELENDT		ULTRAPURE		ULTRAPURE/AUTOTRACE		DRYING ONLY	
Ethoxymer	C_{13}	C_{15}	C_{13}	C_{15}	C_{13}	C_{15}	C_{13}	C_{15}	C_{13}	C_{15}
EO_0	78 (77, 79)	59 (58, 61)	72 (71, 73)	75 (73, 77)	71 (72, 71)	79 (81, 77)	88 (88, 88)	88 (89, 87)	90	99
EO_1	81 (80, 81)	57 (55, 59)	81 (80, 82)	76 (74, 79)	79 (80, 78)	79 (80, 78)	86 (87, 85)	92 (92, 92)	97	102
EO_2	89 (91, 88)	69 (68, 70)	91 (93, 90)	79 (78, 81)	91 (92, 90)	80 (84, 77)	87 (87, 86)	97 (95, 98)	97	100
EO_3	94 (92, 96)	77 (74, 79)	98 (98, 99)	87 (86, 89)	94 (94, 94)	88 (90, 86)	95 (93, 97)	99 (98, 100)	99	99
EO_4	99 (99, 100)	80 (80, 81)	99 (99, 100)	90 (90, 90)	98 (98, 98)	88 (92, 83)	99(98, 101)	95 (96, 93)	96	102
mean $EO_{0\text{-}4}$	**88**	**68**	**88**	**81**	**87**	**83**	**91**	**94**	**96**	**100**
EO_5	101 (100, 101)	83 (83, 83)	102 (102, 101)	96 (95, 97)	99 (98, 99)	100 (103,96)	102 (104, 101)	99 (99, 99)	100	102
EO_6	100 (100, 100)	86 (83, 89)	102 (101, 103)	92 (91, 92)	100 (99, 101)	94 (97, 92)	106 (104, 107)	103 (103, 104)	102	100
EO_7	100 (100, 100)	90 (91, 89)	104 (103, 106)	99 (97, 100)	102 (101, 103)	95 (96, 95)	100 (100, 101)	98 (98, 98)	100	98
EO_8	101 (102, 101)	94 (96, 92)	104 (101, 107)	99 (102, 96)	104 (104, 104)	104 (106, 103)	106 (105, 107)	93 (94, 92)	101	98
EO_9	102 (102, 101)	93 (93, 94)	104 (103, 106)	101 (99, 104)	104 (104, 104)	106 (104, 108)	97 (94, 100)	104 (102, 105)	104	100
EO_{10}	100 (98, 101)	94 (94, 94)	106 (107, 105)	104 (105, 104)	108 (109, 106)	105 (107, 102)	110 (111, 110)	97 (96, 97)	97	102
EO_{11}	105 (108, 102)	98 (97, 98)	105 (105, 105)	102 (101, 104)	104 (106, 102)	103 (104, 102)	107 (107, 108)	101 (102, 101)	101	104
EO_{12}	102 (104, 100)	99 (98, 99)	111 (111, 111)	110 (109, 111)	109 (107, 111)	107 (107, 107)	105 (104, 105)	102 (101, 103)	100	102
EO_{13}	106 (109, 103)	95 (98, 93)	109 (110, 108)	108 (105, 111)	107 (107, 106)	106 (105, 106)	115 (116, 113)	93 (92, 94)	104	98
EO_{14}	101 (99, 103)	96 (97, 94)	107 (107, 106)	100 (103, 97)	104 (100, 108)	101 (100, 102)	109 (109, 108)	106 (102, 109)	100	105
EO_{15}	101 (102, 100)	96 (96, 97)	106 (106, 106)	107 (108, 107)	106 (107, 105)	102 (102, 103)	102 (105, 99)	109 (104, 113)	98	102
EO_{16}	107 (108, 105)	99 (100, 97)	109 (110, 108)	105 (103, 108)	109 (107, 111)	107 (109, 105)	96 (97, 96)	101 (103, 100)	100	101
EO_{17}	104 (105, 104)	98 (98, 99)	109 (111, 107)	104 (104, 103)	102 (100, 104)	105 (106, 104)	112 (110, 114)	104 (104, 103)	100	99
EO_{18}	103 (104, 101)	100 (98, 103)	105 (101, 109)	106 (106, 107)	107 (103, 111)	102 (104, 100)	105 (109, 102)	100 (95, 105)	94	105
EO_{19}	106 (104, 107)	96 (95, 97)	109 (109, 108)	103 (99, 106)	110 (108, 112)	102 (104, 101)	108 (104, 111)	104 (100, 109)	98	100
EO_{20}	106 (108, 104)	101 (95, 106)	114 (113, 115)	102 (96, 109)	107 (100, 114)	109 (111, 107)	95 (89, 101)	106 (106, 106)	98	95
mean $EO_{5\text{-}20}$	**103**	**95**	**107**	**102**	**105**	**103**	**105**	**101**	**100**	**101**

Table 7.2 T-test results calculated in Excel™ for $C_{15}EO_{0-4}$ ethoxylate recovery. Comparison of different media types.

	Drying only	*Ultrapure*	*Drying only*	*Stream*	*Ultrapure*	*Stream*
Mean	100.4	82.8	100.4	68.4	82.8	68.4
Variance	2.3	22.7	2.3	106.8	22.7	106.8
Observations	5	5	5	5	5	5
t Stat	7.9		6.9		2.8	
t Critical two-tail	2.3		2.3		2.3	

7.3 Initial method applied to real samples

Although there were some low SPE recoveries in the data in Table 7.1, as proved statistically for $C_{15}EO_{0-4}$ in Table 7.2, overall the method was thought to be good enough to apply to spiked effluent samples at more realistic environmental concentrations. The full suite of AEs, C_{12} to C_{18}, contained in the commercial samples (Section 5.1.2) was used in this work, which was carried out on the Autotrace. Elution with 5 mL of methyl t-butyl ether (MTBE), a less polar solvent, was included in addition to the 10 mL of methanol used in Section 7.1, in an attempt to improve recovery of the more hydrophobic AEs.

SPE recovery data for total AE spiked at 1200 μg/L and 120 μg/L in final effluent from the site sewage treatment works at Unilever Colworth is shown in Tables 7.3 and 7.4, respectively. Both tables clearly show some unacceptably low recoveries, which appear worst for the more hydrophobic species. The problem is highlighted in the mean recovery of $C_{16}EO_{0-4}$ and $C_{18}EO_{0-4}$ which are only 30% and 22%, respectively in Table 7.3 and 24% and 7%, respectively in Table 7.4. Slight problems with recovery as seen in Table 7.1 have been exaggerated in this work for two reasons. Firstly the full suite of AEs has a wider range of polarity of ethoxymers, when dealing with a sample containing C_{12} to C_{18} AE compared to the C_{13} and C_{15} AE in Lutensol A07. This is demonstrated by the lower recoveries for the most hydrophobic AEs. Secondly the problem is worse when moving to a lower concentration, for example $C_{18}EO_{0-4}$ mean recovery drops from 22 to 7 % for AE spiked at 1200 and 120 μg/L respectively. This is the expected scenario if sites of hydrophobic loss were present and/or hydrophobic AEs were more strongly retained on the SPE, both would lead to a bigger percentage loss in the low spikes. The problems were probably due to the following:

- The elution solvent used was not strong enough to elute the less polar ethoxylates.
- There were possible losses on glassware and also sample flow path, including lines and valves on the Zymark autotrace.

These poor recoveries suggested the method could be optimised much further.

A systematic approach was required to look into the shortfalls observed. In the experiments that follow particular attention was paid to the analysis of C_{18} AEs which had shown the worst recoveries. At the same time C_{12} AEs were also analysed with the idea that if the most non-polar and polar AEs were optimised then all other AEs falling in between must also work. This approach would lead to a much simpler data analysis with fewer extracted ions needing to be considered. The method optimised by this approach could be validated by subsequent analysis of the full suite of compounds. The following section contains details of this investigation.

7.4 Systematic approach to optimisation of SPE

7.4.1 SEQUENTIAL ELUTION WITH DIFFERENT SOLVENTS OF INCREASING SOLVENT STRENGTH

In high performance liquid chromatography (HPLC) and SPE, solvents are often listed in order of elution strength starting with water as a weak solvent through to hexane which is a strong solvent. A sequential elution scheme was tested, using selected solvents, with each fraction analysed to examine the AE content. This work focussed on recoveries of low EO C_{12} and C_{18} species only. Solvents were selected in this investigation based on the properties listed in Table 7.5. Two parameters are shown, elution strength for adsorption chromatography on silica (ε^0) and polarity (P'). It should be noted that increases in eluent strength and decrease in polarity do not follow the same order for the solvents listed.

Table 7.3. Percent recovery of 1200 µg/L total AE spiked into final effluent. Results quoted as mean % (individual replicates), n = 2. Conditions- automated extraction (Autotrace) using a C_2 500 mg/6 mL Isolute cartridge, followed by elution with 10 mL of methanol and 5 mL of MTBE.

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	48 (46, 49)	50 (50, 50)	50 (51, 48)	37 (38, 37)	17 (18, 17)	15 (15, 14)
EO_1	56 (55, 57)	61 (62, 60)	42 (47, 38)	39 (38, 38)	20 (19, 21)	17 (18, 16)
EO_2	66 (65, 67)	73 (73, 73)	52 (51, 53)	50 (51, 49)	27 (26, 27)	19 (18, 19)
EO_3	78 (75, 81)	78 (79, 78)	70 (69, 70)	61 (60, 62)	36 (37, 34)	25 (26, 25)
EO_4	81 (81, 81)	84 (83, 85)	76 (78, 73)	70 (69, 72)	50 (51, 49)	33 (31, 35)
Mean $EO_{0\text{-}4}$	**66**	**69**	**58**	**52**	**30**	**22**
EO_5	85 (84, 87)	80 (79, 81)	87 (86, 88)	77 (75, 79)	61 (59, 63)	35 (35, 35)
EO_6	82 (82, 81)	89 (89, 88)	87 (87, 86)	80 (78, 82)	70 (68, 71)	41 (40, 42)
EO_7	82 (86, 79)	85 (88, 82)	89 (85, 92)	78 (76, 80)	76 (78, 74)	45 (44, 47)
EO_8	81 (79, 84)	88 (87, 88)	81 (79, 83)	81 (77, 86)	77 (76, 78)	48 (48, 48)
EO_9	90 (93, 87)	92 (90, 95)	86 (86, 87)	87 (87, 87)	78 (81, 75)	51 (52, 49)
EO_{10}	89 (89, 90)	89 (90, 89)	89 (85, 92)	78 (78, 77)	84 (82, 85)	56 (55, 57)
EO_{11}	92 (94, 90)	89 (88, 89)	89 (89, 88)	86 (84, 88)	83 (83, 82)	52 (54, 51)
EO_{12}	89 (89, 90)	81 (77, 86)	90 (87, 93)	90 (89, 91)	77 (77, 77)	59 (58, 59)
EO_{13}	87 (87, 86)	85 (82, 88)	96 (98, 93)	83 (79, 87)	88 (92, 85)	63 (64, 62)
EO_{14}	85 (87, 84)	88 (87, 90)	100 (97, 102)	97 (103, 92)	80 (82, 78)	65 (63, 67)
EO_{15}	94 (93, 95)	89 (90, 88)	90 (95, 85)	88 (88, 87)	80 (82, 79)	67 (66, 69)
EO_{16}	89 (88, 89)	88 (88, 87)	89 (88, 91)	93 (90, 96)	78 (78, 78)	71 (71, 71)
EO_{17}	91 (93, 90)	88 (86, 90)	85 (87, 82)	93 (93, 93)	84 (84, 85)	72 (73, 70)
EO_{18}	90 (89, 91)	85 (84, 86)	89 (88, 91)	97 (88, 107)	83 (87, 79)	69 (74, 63)
EO_{19}	95 (97, 93)	85 (85, 86)	89 (89, 90)	108 (104, 112)	92 (96, 87)	76 (76, 76)
EO_{20}	92 (92, 91)	78 (76, 80)	86 (88, 85)	111 (112, 110)	83 (84, 82)	79 (79, 79)
Mean $EO_{5\text{-}20}$	**88**	**86**	**89**	**89**	**80**	**59**

Table 7.4. Percent recovery of 120 µg/L total AE spiked into final effluent. Results quoted as mean % (individual replicates), n = 2. Conditions- automated extraction (Autotrace) using a C_2 500 mg/6 mL Isolute cartridge, followed by elution with 10 mL of methanol and 5 mL of MTBE.

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	3 (4, 2)	23 (29, 17)	40 (39, 41)	23 (26, 20)	37 (29, 45)	8 (7, 8)
EO_1	33 (33, 34)	38 (31, 46)	26 (22, 31)	30 (40, 20)	11 (14, 8)	6 (3, 9)
EO_2	78 (68, 87)	53 (51, 55)	41 (32, 51)	34 (32, 36)	20 (23, 17)	7 (5, 9)
EO_3	67 (58, 76)	61 (59, 63)	56 (60, 52)	44 (39, 49)	21 (20, 22)	7 (7, 8)
EO_4	69 (64, 74)	78 (74, 83)	59 (55, 64)	41 (36, 45)	30 (30, 29)	9 (9, 9)
Mean EO_{0-4}	**50**	**51**	**45**	**34**	**24**	**7**
EO_5	78 (77, 79)	89 (85, 93)	52 (50, 54)	49 (46, 52)	29 (29, 28)	11 (12, 11)
EO_6	67 (67, 67)	73 (73, 73)	70 (70, 69)	47 (48, 46)	32 (30, 34)	14 (14, 15)
EO_7	54 (53, 54)	92 (89, 94)	64 (59, 68)	44 (39, 49)	39 (40, 38)	21 (21, 21)
EO_8	61 (62, 60)	81 (78, 84)	69 (65, 74)	51 (51, 50)	44 (40, 48)	24 (23, 26)
EO_9	60 (59, 60)	76 (77, 76)	79 (76, 82)	46 (39, 54)	43 (43, 44)	29 (26, 32)
EO_{10}	81 (76, 85)	89 (90, 88)	72 (70, 74)	54 (51, 58)	50 (46, 53)	34 (33, 35)
EO_{11}	81 (80, 81)	82 (85, 79)	69 (65, 72)	62 (62, 62)	53 (52, 54)	43 (41, 46)
EO_{12}	77 (78, 76)	87 (84, 91)	78 (77, 79)	76 (66, 86)	53 (49, 57)	48 (45, 51)
EO_{13}	80 (81, 80)	82 (78, 86)	90 (85, 95)	68 (67, 70)	64 (57, 70)	44 (42, 45)
EO_{14}	81 (85, 77)	85 (84, 87)	95 (99, 92)	81 (78, 83)	82 (84, 80)	53 (54, 53)
EO_{15}	90 (91, 89)	87 (89, 85)	94 (90, 98)	39 (35, 43)	67 (62, 72)	52 (47, 58)
EO_{16}	89 (87, 92)	82 (76, 89)	106 (92, 120)	80 (79, 81)	71 (69, 72)	55 (53, 58)
EO_{17}	93 (89, 97)	97 (92, 102)	96 (93, 98)	93 (86, 100)	83 (82, 84)	53 (48, 59)
EO_{18}	89 (90, 88)	64 (59, 69)	97 (99, 96)	79 (85, 72)	78 (74, 81)	61 (59, 63)
EO_{19}	100 (102, 97)	82 (85, 79)	108 (109, 107)	115 (79, 150)	81 (79, 83)	69 (67, 71)
EO_{20}	85 (87, 82)	80 (74, 85)	102 (88, 116)	100 (94, 106)	88 (79, 96)	64 (60, 68)
Mean EO_{5-20}	**79**	**83**	**84**	**68**	**60**	**42**

Table 7.5. Solvent properties of elution solvents.

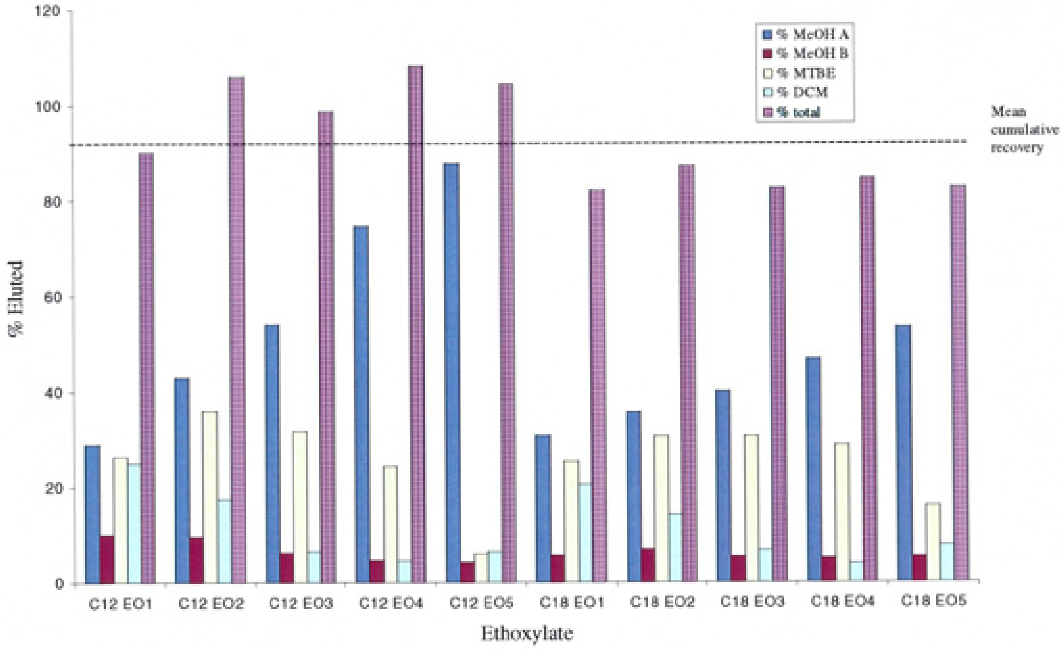
Eluent strength for adsorption chromatography on silica and polarity [5]

Solvent	**Eluent strength (ε^0)**	**Polarity (P')**
Hexane	0.01	0.1
Dichloromethane (DCM)	0.30	3.1
MTBE	0.48	2.5
Methanol	0.70	5.1

A 500 mg C_8 cartridge from Phenomenex (Macclesfield, UK) was conditioned with methanol and water. With the cartridge still full with Ultrapure water, 60 μg of total AE in methanol was spiked into the cartridge and allowed to pass through the stationary phase. This was then thoroughly dried by pulling air through the cartridge under vacuum for approximately 1 hr. Sequential elution with different solvents and collection in separate vials was then carried out. The eluents were then taken through the normal blow down and derivatisation procedure (see Section 5.4.1) to look at the AE content of each aliquot. The following 5 mL fractions in the order listed were collected: methanol (2 x 5 mL aliquots), MTBE, DCM and hexane. The results of elution with the chosen solvents can be seen in Figure 7.1. It can be seen that methanol alone will not elute off all the AEs. This effect is demonstrated in the data for $C_{18}EO_{1-5}$, where the second elution in methanol does not contribute significantly to the eluted AEs. For these AEs MTBE and DCM elution contribute significantly to the total eluted. In the data for $C_{18}EO_1$ there is approximately a 30 % contribution each from methanol, MTBE and DCM. In particular there was a large amount of AE which was eluted by the DCM after sequential elution with methanol and MTBE, suggesting that this solvent must be included in the elution scheme. However the use of hexane subsequent to DCM did not elute anything further. This was the least polar/stongest solvent used and it appeared that it would not be necessary for elution of these compounds. For AEs with a higher EO number than those displayed in Figure 7.1, methanol alone was efficient in elution from the cartridge. The cumulative recovery of the combined eluents is also plotted in Figure 7.1. A mean cumulative recovery for the ethoxylates plotted of 93 ± 11 %, is also denoted by the dashed line.

Figure 7.1: Sequential elution of AEs with different solvents using a C_8 500 mg from Phenomenex, spiked with 60 µg of total AE.

Conditions- eluted with 5 mL aliquots of solvents in the following order: MeOH A, MeOH B, MTBE and DCM.



Data for $C_{12}EO_0$ and $C_{18}EO_0$ (free alcohols) was not included because of the presence of these compounds in solvents supplied by Fisher (Loughborough, UK), used at the time of this experiment. This was thought to be due to the practice of reusing solvent bottles with surfactant residues introduced in the cleaning process.

The data in Table 7.1 shows that the % eluted by DCM, the strongest solvent, increases as the EO number decreases. The next experiment was carried out for two reasons: firstly to confirm that the methanol/DCM/MTBE could be applied as a one step elution mix and secondly to see if the combination of methanol and DCM alone would be equally effective. A total volume of elution solvent of 16 mL was used in this work to be compatible with vials and volumes used on the automated SPE workstation in later work. Duplicate cartridges for each test were conditioned, spiked and dried as before and then eluted as follows:

- Two cartridges were eluted with methanol (8 mL), MTBE (4 mL) and DCM (4 mL).
- Two cartridges were eluted with methanol (8 mL), DCM (8 mL)

The average % recoveries are compared in Table 7.6:

Table 7.6 Comparison of elution of C_8 500 mg cartridges from Phenomenex, spiked with 60 µg of total AE, using methanol/MTBE/DCM (8 mL/ 4 mL/ 4 mL) and methanol/DCM (8 mL/ 8 mL).
Results quoted as mean % (individual replicates), n = 2

SOLVENT	% RECOVERY	
	C_{12}	C_{18}
Methanol/MTBE/DCM	94 (94, 94)	79 (78, 79)
Methanol/DCM	94 (93,95)	79 (77, 82)

The data in Table 7.6 suggested that the MTBE could be replaced by using an equivalent amount of DCM. However more detailed inspection of individual ethoxylate recoveries and also the fact that MTBE was the least polar (see Table 7.5) lead to the conclusion that it was appropriate to keep the three solvent mixture at this stage.

The optimised elution mix for any future work would be methanol/MTBE/DCM (2/1/1, v/v/v) with a total volume of approximately 15 mL. The actual volume of solvent used could be optimised at a later date, a value which would also be affected by the amount of sorbent used. However this combination of solvents appeared to offer the right selectivity for elution of the lower C_{18}EOs, an area which had been highlighted for improvement in the preliminary work carried out in Section 7.1. The next stage would use the improved elution scheme to investigate if AEs were not being recovered in other parts of the process.

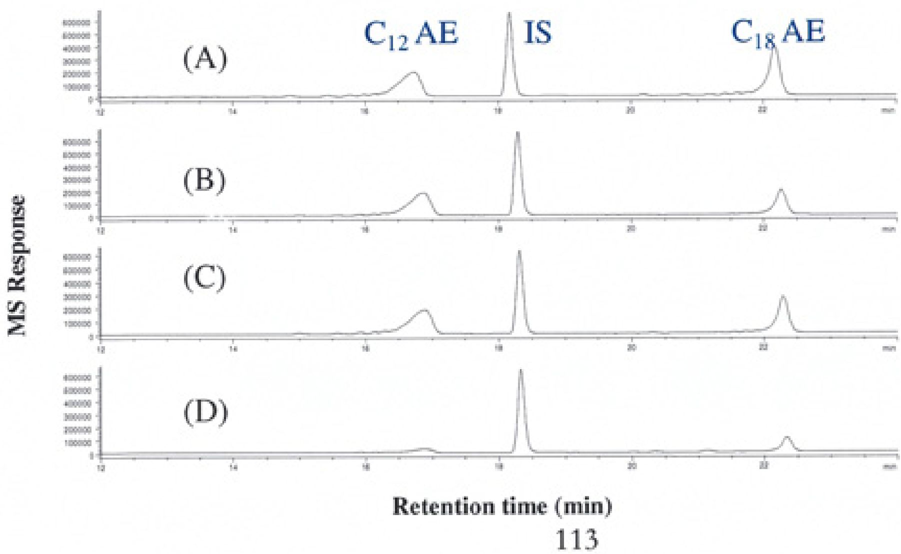
7.4.2 LOSS OF HYDROPHOBIC AEs IN THE LOADING PROCESS

The next area for investigation was the loss of hydrophobic species in the loading process. An experiment was designed to monitor AE recovery with and without the addition of methanol to the sample being loaded. The amount of residual AE on glassware was also analysed by incorporating an additional extraction step. The work was carried out on the Zymark Autotrace. To evaluate the losses, duplicate 60 μg total AE spikes in 500 mL of Ultrapure water were loaded onto an Isolute C_8 1g cartridge with and without the presence of methanol (4% v/v) in the water. The cartridge choice (size and supplier) was different from that used in Section 7.2.1, however this was the preferred way forward for the project and was not thought to compromise either the work done so far or the current experiment. Methanol was added to reduce the interaction of the hydrophobic AE with glassware and tubing, a commonly used approach in SPE. A re-extraction of the bottle for the methanol containing samples was also included to see if any AE were left. The duplicates were taken through separate procedures described below:

Procedure (i): One empty bottle was extracted with 20 mL of methanol before diluting to 100 mL with Ultrapure water. This 20 % v/v methanol solution was then loaded onto the same SPE cartridge.

Procedure (ii): One bottle was extracted with 15 mL of elution solvent (Section 5.3.1) which was transferred to a vial, taken to dryness and derivatised.

Figure 7.3 Single ion monitoring (SIM) data showing hydrophobic losses of AEs in the loading process. Extraction of 60 μg of total AE, spiked into a bottle containing 500 mL of water, was carried out on a C_8 1 g cartridge from Isolute and eluted with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).
(A) 60 μg/mL of total AE standard using the SIM method (Section 5.5.2)
(B) SPE extract, from a sample loaded with no methanol
(C) SPE extract, from a sample loaded with 4% methanol
(D) AE obtained from re-extraction of bottle [Procedure (i)]

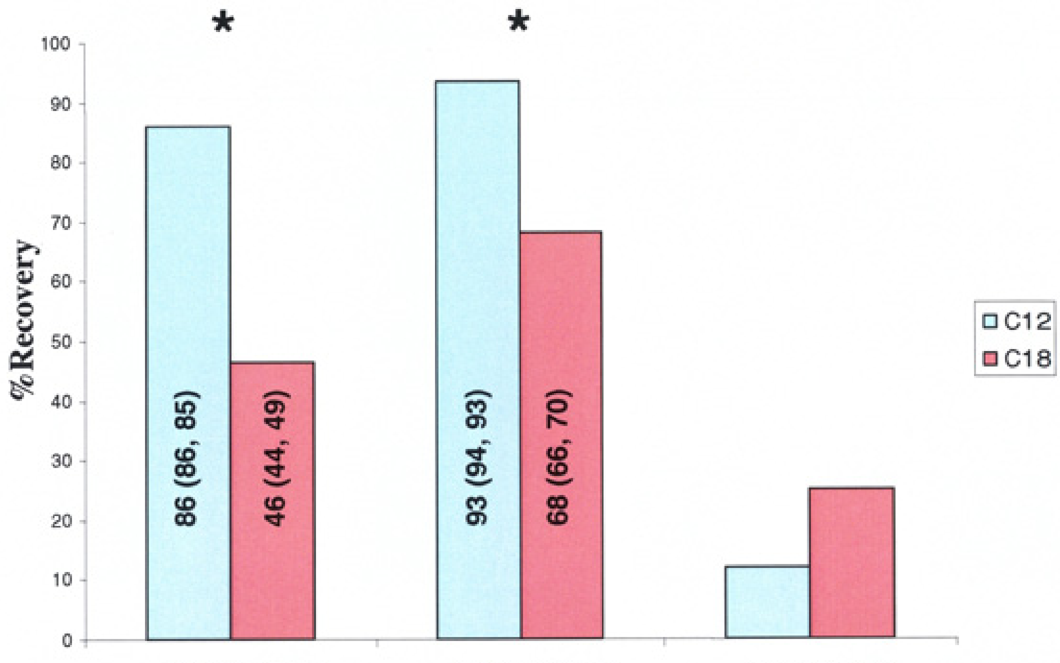


The data in Figure 7.3 (C) clearly demonstrates a better recovery of C_{18} with the inclusion of methanol in the sample on loading. However Figure 7.3 (D) shows that there is still C_{18} coming from the re-extraction of the bottle. This data has also been displayed graphically in Figure 7.4. This data shows an increase in recovery of C_{18} from 46 % with no methanol to 68 % with methanol (4 % v/v) added. The re-extraction accounts for 25 % of the C_{18} spiked into the bottle.

In Figure 7.4 the data labelled "re-extraction" was from procedure (i). Similar results were obtained from procedure (ii), although with a slightly lower C_{18} recovery. This suggested that extraction of the bottle only with solvent does not remove any hydrophobic AE associated with tubing in the flow system. Procedure (i) would be a more efficient method for future use. From the data presented in Figure 7.4, C_{18} recovery would have been >90 % if this approach had been combined into one extract.

Figure 7.4 Hydrophobic losses in the loading process
mean % (individual replicates), n = 2*, otherwise n = 1

Experimental conditions- 60 μg of AE in 500 mL of water, with and without the addition of methanol, extracted on C_8 1 g cartridges from Isolute and eluted with 15 mL of methanol/DCM/MTBE (2/1/1, v/v). The bottle wash is AE re-extracted from glassware of the 4 % methanol sample.



7.4.3 *COMPARISON OF DIFFERENT PHASES WITH THE 'RE-EXTRACTION' METHODOLOGY*

Replicate samples, consisting 60 µg AE spiked in 500 mL Ultrapure water with methanol added at 4 % v/v, were loaded onto selected SPE cartridges to be evaluated. After loading, a 20 mL methanol re-extraction, diluted to 100 mL with water was loaded onto the same cartridge. Because of the manual intervention required and also the number of cartridges to be evaluated, this work was carried out on the Vac-Elut manifold.

The following silica based cartridges were evaluated: C_2/500 mg, C_2/1 g, C_8/1 g, C_{18}/1g (Isolute) and also the following polymeric phases: RDX/500 mg, Oasis HLB/500 mg from Waters (Elstree, UK), ENV+/200 mg and 101/200 mg (Isolute).

Figure 7.5. Performance of different cartridges.
mean % (individual replicates), n = 2*, otherwise n = 1

Experimental conditions (Vac Elut)- 60 µg of AE in 500 mL of water, with the addition of methanol (4 % v/v), were loaded on different cartridges. A 20 mL methanol re-extraction of the bottle (diluted to 100 mL with water before loading) and elution with 15 mL of methanol/DCM/MTBE (2/1/1, v/v) was carried out.

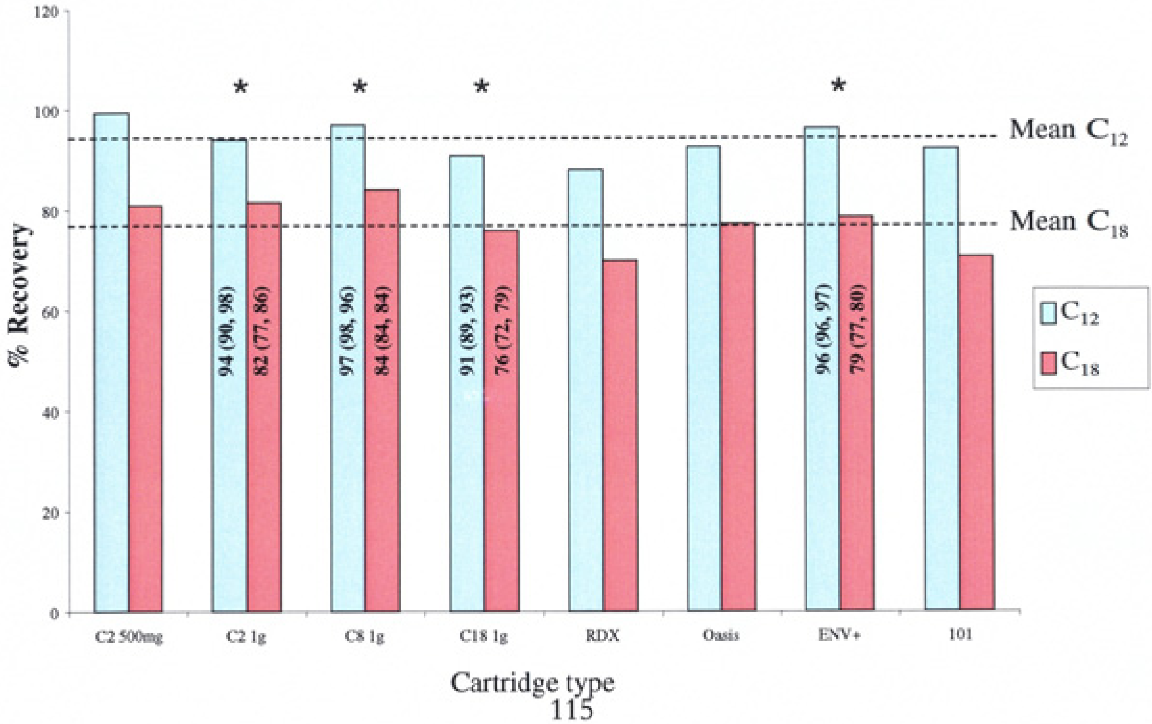


Figure 7.5 shows minor differences in recovery dependent on phase type. The C_8/1g cartridge from Isolute was selected for any future work on the basis of its overall better performance when considering both C_{12} and C_{18} recovery. Looking at the data in Figure 7.5, however, other cartridges where the recovery of both C_{12} and C_{18} are greater than the mean value, e.g. ENV+, could equally well have been selected for further evaluation.

7.4.4 *COMPARISON OF ELUTION SOLVENT MIXES USED IN THE LITERATURE WITH THAT DEVELOPED SO FAR*

Using the C_8 1 g Isolute cartridges, 60 µg of AE in 500 mL of Ultrapure spikes were analysed on the Zymark Autotrace, stopped after the drying stage, before manually eluting with three different solvent systems. Six samples in total, duplicates for each solvent system were analysed. The method developed on the Vac Elut manifold had the following key features to allow transfer to the Autotrace.

The spiked samples were loaded with the addition of methanol (4 % v/v) as normal. After loading of the first 500 mL the system was paused to allow a re-extraction to take place. This consisted of adding 20 mL of methanol to each bottle, shaking and making up to 100 mL with Ultrapure water before loading on the Autotrace. After a drying time of one hour the method stopped so that the different elution schemes, listed below, could be applied to the relevant cartridges.

Elution Scheme A: Methanol: MTBE: DCM (2:1:1, v/v/v) 3 x 5 mL aliquots combined

Elution Scheme B: Methanol: ACN (1:2, v/v), 7.5 mL, methanol:DCM (1:4), 7.5ml aliquots combined [6].

Elution Scheme C: Hexane: DCM (1:4, v/v) 2x 5 mL aliquots combined [7].

Elution SchemeD: Methanol: DCM (9:1, v/v) 2 x 5 mL aliquots combined [7].
Elution scheme A is that developed so far in this work. Elution scheme B was taken from Krogh et al. [6], in which C_{18} 1g cartridges were tested in comparison to

polymeric phases. Elution schemes C and D were taken from Petrovic et al. [7] and AEs were reported to elute mainly in fraction C in a sequential elution scheme from a C_{18} sorbent. However both aliquots were analysed for AE in this experiment. In all cases the extracts were taken to dryness before carrying out derivatisation (Section 5.4.1) and LC/MS (Section 5.5.2). The results obtained are summarised in Table 7.7 and showed that elution scheme A, methanol/MTBE/DCM, was comparable to or superior to the schemes used in the literature. As suspected the AE eluted in fraction D for the work carried out by Petrovic et al. [7].

Table 7.7 Different elution schemes.
Results quoted are mean % (individual replicates), n = 2

Experimental conditions (Autotrace)- 60 µg of AE in 500 mL of water, with the addition of methanol (4 % v/v), were loaded on C_8 1 g Isolute cartridges. A 20 mL methanol re-extraction of the bottle (diluted to 100 mL with water before loading) was carried out before manual elution with:
***A:* Methanol: MTBE: DCM (2:1:1, v/v/v), 3 x 5 mL aliquots combined**
***B:* Methanol: ACN (1:2, v/v), 7.5 mL and methanol: DCM (1:4), 7.5ml, aliquots combined**
***C:* Hexane: DCM (1:4, v/v), 2x 5 mL aliquots combined**
***D:* Methanol: DCM (9:1, v/v), 2 x 5 mL aliquots combined**

Elution scheme	% Recovery of AE	
	C_{12}	C_{18}
A	97 (100,95)	70 (70,69)
B	92 (93,91)	62 (63, 62)
C	1 (2,1)	3 (3, 3)
D	95 (94,95)	70 (69, 71)

For the work carried out with elution scheme A, as the same methodology had previously been used in Section 7.4.3, a comparison of Autotrace and Vac Elut loaded samples could be made. The results in Figure 7.5 showed a mean C_{18} recovery of 84 % for the C_8 1g cartridge on the Vac Elut, which is higher than the

70 % obtained in Table 7.7 for the Autotrace loaded samples. Given the small number of replicates analysed in each determination the significance of this will have to be proven in further work. However it could be postulated that this was due to a longer sample flow path in the Autotrace and thus greater potential adsorption of hydrophobic AEs.

7.4.5 ADDITION OF 20 % V/V METHANOL PRIOR TO LOADING AND COMPARISON OF LOAD FLOWS

Previous work had shown that 20 % v/v methanol in Ultrapure water allowed AE, in a re-extraction procedure, to be retained onto a C_8 cartridge. By adding this percentage of methanol prior to loading the need for a bottle re-extraction may not be necessary. This would also help with the ultimate goal of fully automating the extraction procedure. This work was done on the Autotrace system using normal conditions except with a load flow of 1 mL/min. 60 μg of AE were added to 400 mL of Ultrapure water then 100 mL of methanol was added. Blank Ultrapure water with the same amount of methanol was also analysed.

The results shown in Table 7.8 are from duplicate extractions and were blank corrected. The Ultrapure water blank was significant for the C_{18} (equivalent to 8 % AE), suggesting this contribution was from the increased amount of methanol used. The main area of concern however from the data in Table 7.8 was the very low C_{18} recovery. The hydrophobic loss appeared more pronounced at the very slow loading rate, suggesting that increased contact time with bottle and tubing can increase losses. The work was repeated with an Autotrace load flow of 15 mL/min and comparing C_{18} recovery at different load flows in Table 7.8 confirmed this to be the case.

Table 7.8 Recovery of AE with 20% v/v methanol and load rates of 1 and 15 mL/min.

Results quoted are mean % (individual replicates), n = 2

Experimental conditions (Autotrace)- 60 μg of AE in 400 mL of water, with the addition of methanol (100 mL), were loaded on C_8 1 g Isolute cartridges, before drying with nitrogen (1 hr) and elution with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).

Load flow (mL/min)	**% Recovery of AE**	
	C_{12}	C_{18}
1	90 (92, 88)	40 (42, 37)
15	100 (102, 99)	60 (60, 59)

The next stage in optimisation would be to determine whether an optimum flow rate between 1 and 15 mL/min could improve C_{18} recovery further or whether the amount of methanol added could be increased, without causing losses due to SPE breakthrough. The latter was looked at first.

7.4.6 INCREASING AMOUNTS OF METHANOL ADDED TO THE SAMPLE

The Autotrace system was used with a flow rate of 10 mL/min. Differing amounts of methanol were added to 60 μg spikes in a total volume of Ultrapure water and methanol equalling 500 mL. Recoveries are presented in Table 7.9.

The data shows a marked increase in recovery of C_{18} AE from 68 % to 110% with methanol added at 20% v/v and 40% v/v, respectively. The figure of 110 % recovery can be explained by the presence of AEs in the solvent obtained from Fluka Ltd (Gillingham, UK). Typical blank levels are also quoted in the table. These results however were very encouraging and clearly showed that higher than expected amounts of methanol can be added to the sample without breakthrough of the AE. The C_{12} component remained optimised as well as achieving good recovery of the more hydrophobic C_{18} component.

Table 7.9 Effect of the amount of methanol added prior to loading SPE sample.

Experimental conditions (Autotrace)- 60 μg of AE in water, with the addition of varying amounts of methanol (total volume of methanol and water = 500 mL), were loaded on C_8 1 g Isolute cartridges at 10 mL/min, before drying with nitrogen (1 hr) and elution with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).

Methanol added (%)	% Recovery of AE	
	C_{12}	C_{18}
5	91	66
10	87	61
15	99	65
20	102	68
25	101	73
30	100	84
35	96	96
40	96	110
45	95	105
(Blank 20%)	1	2
(Blank 45%)	0	15

7.5 Optimised methodology applied to influent and effluent samples

The methodology developed in Section 7.4 was now applied to environmental levels of AEs in effluent and influent samples. The key features of the SPE method are summarised as follows but full details can be found in Chapter 5: methanol (40 % v/v) was added to samples prior to loading to improve hydrophobic AE recovery, C_8 1g (Isolute) was determined to be the most efficient SPE cartidge with elution using methanol/MTBE/DCM (2/1/1, v/v/v), total volume of 15 mL. Methanol supplied by Rathburn Chemicals Ltd. eliminated the blank problems observed in method development. The addition of methanol prior to loading allowed the procedure to be fully automated on the Autotrace system.

Effluent and influent samples were taken from Broardholme sewage treatment plant (Wellingborough, UK) in borosilicate glass bottles which had previously been rinsed with Ultrapure water and methanol. Samples were held at 4°C and preconcentrated no later than 24 hr after sampling. Effluent was filtered through a Whatmann GF/C filter (Maidstone, UK) until a volume of approximately 4 L was collected. Six 600 mL aliquots were dispensed into 1 L bottles, three were spiked at 100 µg/L total AE and three were left unspiked. To each of the six samples 400 mL of methanol were added. 120 mL of influent was thoroughly mixed with 1680 mL of Ultrapure water and divided into six 300 mL aliquots. Three of these were spiked at 1 mg/L of total AE and three were left unspiked. To all six influent samples 200 mL of methanol were added. Extraction and analysis by LC/MS were carried out according to procedures in Chapter 5 and typical total ion chromatograms (TICs) and extracted ion chromtograms (EICs) can be found in Chapter 6.

Table 7.10. Recovery of 100 µg/L of total AE spiked into final effluent Values quoted are mean (%RSD), (n = 3).

Experimental conditions (Autotrace)- 600 mL final effluent samples (spiked and unspiked), with the addition of 400 mL of methanol, were loaded on C_8 1 g Isolute cartridges at 10 mL/min. The cartridges were dried with nitrogen (1 hr) and eluted with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).

	C_{12}		C_{13}		C_{14}		C_{15}		C_{16}		C_{18}	
EO_0	55	(14)	63	(2)	84	(23)	88	(3)	108	(25)	102	(39)
EO_1	93	(6)	79	(8)	112	(4)	65	(36)	93	(10)	93	(4)
EO_2	110	(10)	93	(11)	116	(7)	95	(9)	100	(2)	90	(3)
EO_3	102	(3)	97	(5)	111	(5)	104	(9)	95	(8)	92	(4)
EO_4	101	(4)	91	(3)	105	(6)	81	(5)	93	(5)	90	(2)
Mean EO_{0-4}	**92**	**(7)**	**85**	**(6)**	**105**	**(9)**	**87**	**(12)**	**98**	**(10)**	**93**	**(10)**
EO_5	101	(4)	88	(5)	104	(8)	89	(4)	100	(3)	98	(2)
EO_6	102	(2)	90	(5)	100	(7)	83	(8)	92	(1)	100	(4)
EO_7	96	(2)	91	(3)	100	(2)	84	(15)	99	(3)	91	(2)
EO_8	102	(2)	89	(4)	101	(1)	74	(18)	98	(6)	91	(3)
EO_9	99	(2)	94	(1)	100	(3)	78	(12)	96	(4)	93	(2)
EO_{10}	96	(1)	96	(3)	92	(6)	79	(6)	95	(5)	93	(3)
EO_{11}	101	(1)	91	(2)	Int	(Int)	96	(2)	90	(3)	91	(3)
EO_{12}	97	(1)	91	(2)	100	(10)	75	(5)	95	(4)	95	(0)
EO_{13}	96	(3)	89	(3)	92	(6)	98	(7)	90	(5)	94	(7)
EO_{14}	91	(7)	98	(5)	87	(5)	92	(7)	93	(4)	96	(6)
EO_{15}	94	(4)	98	(6)	98	(2)	91	(2)	86	(6)	99	(2)
EO_{16}	94	(8)	91	(4)	91	(8)	107	(8)	101	(8)	98	(1)
EO_{17}	100	(7)	113	(5)	85	(8)	92	(6)	96	(9)	95	(2)
EO_{18}	95	(9)	105	(10)	99	(11)	99	(7)	95	(1)	90	(6)
EO_{19}	95	(9)	102	(7)	94	(9)	117	(11)	98	(13)	102	(3)
EO_{20}	94	(2)	82	(18)	90	(7)	76	(10)	111	(14)	90	(11)
Mean EO_{5-20}	**97**	**(4)**	**94**	**(5)**	**96**	**(6)**	**89**	**(8)**	**96**	**(6)**	**95**	**(4)**

Int = isobaric interference

The blank effluent samples were found to have low concentrations of native AE present, but these concentrations were subtracted from AE concentrations measured in spiked samples in calculating recoveries. An internal standard was used to assess derivatisation efficiency and also to confirm that matrix suppression effects were not affecting quantitation. No matrix suppression was observed and results were not corrected for internal standard response. Results reported for recoveries of AE were assessed on the basis of the whole procedure i.e. SPE and the preconcentration / derivatisation step. Losses of the more volatile AE components (e.g. C_{12} and C_{13} free alcohol) were reduced by careful control of the preconcentration step. The calibration standards were not subjected to blow down and hence any evaporative losses.

Table 7.10 shows spiked effluent recoveries. It is apparent that excellent recoveries and % RSD figures were obtained across both the alkyl chain and ethoxymer chain ranges, albeit with some slightly lower recoveries being observed in the C_{12} and C_{13} free alcohols, probably due to evaporative losses. The addition of 40 % methanol to samples prior to loading appears to improve recovery for apolar surfactants. This does not appear to have been used in other recent papers which discuss the optimisation of AE recovery using SPE, and may account for some of the lower recoveries reported for these species [3,6]. The recovery problem as shown in Table 7.4 appears to have been solved by careful selection of elution solvents and prevention of hydrophobic losses.

Table 7.11 Recovery of 1000 μg/L of total AE spiked into final influent
Values quoted are mean (%RSD), (n = 3)

Experimental conditions (Autotrace)- 20 mL influent samples, with the addition of 280 mL of water (spiked and unspiked) and 200 mL of methanol were loaded on C_8 1 g Isolute cartridges at 10 mL/min. The cartridges were dried with nitrogen (1 hr) and eluted with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).

	C_{12}		C_{13}		C_{14}		C_{15}		C_{16}		C_{18}	
EO_0	60	(33)	64	(9)	42	(72)	84	(4)	100	(19)	78	(7)
EO_1	45	(38)	85	(3)	49	(44)	66	(4)	81	(7)	90	(5)
EO_2	34	(109)	89	(1)	88	(25)	107	(12)	100	(10)	88	(6)
EO_3	51	(58)	96	(2)	86	(18)	106	(10)	106	(13)	91	(7)
EO_4	61	(26)	93	(5)	88	(9)	92	(14)	108	(3)	98	(4)
Mean EO_{0-4}	**50**	**(53)**	**86**	**(4)**	**71**	**(34)**	**91**	**(9)**	**99**	**(10)**	**89**	**(6)**
EO_5	74	(16)	89	(2)	93	(11)	98	(8)	112	(4)	98	(5)
EO_6	91	(6)	96	(2)	104	(5)	101	(11)	103	(5)	93	(6)
EO_7	89	(5)	97	(10)	98	(3)	94	(8)	104	(2)	94	(2)
EO_8	99	(2)	97	(7)	113	(3)	99	(7)	99	(7)	92	(2)
EO_9	93	(4)	99	(10)	115	(1)	87	(16)	98	(5)	88	(3)
EO_{10}	98	(3)	92	(2)	107	(6)	95	(6)	102	(4)	98	(2)
EO_{11}	94	(1)	103	(5)	Int	(int)	108	(5)	99	(3)	94	(4)
EO_{12}	97	(2)	94	(6)	106	(7)	100	(22)	93	(4)	100	(1)
EO_{13}	94	(2)	91	(9)	104	(15)	101	(5)	93	(3)	95	(1)
EO_{14}	92	(7)	97	(8)	86	(21)	97	(12)	94	(3)	94	(3)
EO_{15}	90	(4)	105	(2)	90	(8)	96	(10)	90	(8)	98	(3)
EO_{16}	98	(11)	106	(6)	88	(5)	113	(2)	95	(5)	98	(4)
EO_{17}	92	(3)	100	(2)	97	(5)	125	(3)	97	(2)	102	(7)
EO_{18}	107	(7)	100	(3)	103	(10)	125	(25)	104	(7)	105	(3)
EO_{19}	105	(9)	112	(6)	108	(8)	165	(5)	103	(13)	99	(5)
EO_{20}	95	(9)	100	(14)	102	(9)	156	(30)	93	(9)	93	(11)
Mean EO_{5-20}	**94**	**(6)**	**99**	**(6)**	**96**	**(8)**	**110**	**(11)**	**99**	**(5)**	**96**	**(4)**

Int = isobaric interference

Analogous data was obtained for influent samples (Table 7.11). However as a result of the higher native levels of AEs in the influent, a much higher level of spike was required to give a measurable difference in response. A 10-fold dilution, made in derivatisation reagent, was required to bring the blank influent samples and spikes within the calibration range used.

Table 7.12 Concentration of AE found in sewage influent from Broardholme STW (μg/L)

Experimental conditions (Autotrace)- 20 mL influent samples, with the addition of 280 mL of water and 200 mL of methanol, were loaded on C_8 1 g Isolute cartridges at 10 mL/min. The cartridges were dried with nitrogen (1 hr) and eluted with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).

Native AE in influent (μg/L)						
	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	146	106	196	68	110	520
EO_1	127	55	125	19	33	<6
EO_2	157	52	131	23	31	<6
EO_3	213	60	77	30	10	<6
EO_4	203	62	55	35	11	<6
EO_5	169	67	46	32	<6	<6
EO_6	145	47	40	36	<6	<6
EO_7	103	45	32	41	<6	<6
EO_8	90	47	26	38	<6	<6
EO_9	78	43	21	39	<6	<6
EO_{10}	58	42	20	35	<6	<6
EO_{11}	47	35	Int	29	<6	<6
EO_{12}	35	26	8	21	<6	<6
EO_{13}	24	21	7	18	<6	<6
EO_{14}	16	11	7	11	<6	13
EO_{15}	11	<6	6	<6	<6	<6
EO_{16}	<6	<6	<6	<6	<6	<6
EO_{17}	<6	<6	<6	<6	<6	<6
EO_{18}	<6	<6	<6	<6	<6	<6
EO_{19}	<6	<6	<6	<6	<6	<6
EO_{20}	<6	<6	<6	<6	<6	<6
Totals	<1652	<756	<829	<510	<290	<648
Total	**<4685**					

Int = isobaric interference

Native concentrations of AEs in influent and effluent from Broardholme sewage treatment plant were determined using the procedures described above (Tables 7.12 and 7.13, respectively). Individual AE concentrations were calculated using characterisation data in Table 6.9, giving % w/w of each ethoxymer present in the commercial mixtures used in this study. It was noted in the influent samples (Table 7.12) that significant levels of EO_0- EO_{15} were observed for C_{12} to C_{15} species, which is consistent for the average structure $C_{13.3}EO_{8.2}$ for European use [8] whereas C_{16} and C_{18} distributions were predominantly free alcohol. This is consistent with previous published monitoring data [3], where it was postulated that AE distributions may have contributions from related surfactants. In the case of effluent samples (Table 7.13) it is evident that the treatment works at Broardholme is functioning effectively in relation to AE removal, as observed AE concentrations were approximately 1000-fold lower than those observed in influent samples. Total AE in the influent samples was approximately 5000 μg/L whilst the concentration in effluent samples was approximately 7 μg/L. These figures include the contribution of the method detection limit (MDL) values for components not detected, which were corrected for the overall concentration step used for each type of sample. An MDL of 0.02 μg/L for each ethoxylate component was estimated from the data. This was obtained by utilising the Poisson-like distribution of ethoxylates within the commercial samples, which results in certain ethoxylates being present at low concentrations in the standard mix and effluent spikes, and peak to peak signal to noise ratios calculated within the ChemStation software. A signal to noise ratio of 3 was then used to define the MDL for such components and this was applied as a general MDL for all ethoxylates based on the similar ionisation response of the derivatives across the mass range. The extract from SPE was divided into two equal aliquots, with one being taken through the procedure described in Chapter 5. The other aliquot was retained for additional analysis if required. An MDL of 0.04 μg/L is quoted in Table 7.13 as a result.

This MDL of 0.02 μg/L per individual ethoxylate equates to an MDL of approximately 2.5 μg/L of total AE (126 species analysed). The predicted no effect concentration for AE has been calculated as 110 μg/L [8] and the method appears to be more than capable of providing data suitable for risk assessment and monitoring below this concentration.

The presence of native AE surfactants in the influent and effluent samples may also have contributed to the reduced precision and recoveries obtained for certain components in the spiked samples (Tables 7.10 and 7.11). Derivatised sample extracts were found to be stable for at least 18 days.

Table 7.13. Concentration of AE found in sewage effluent from Broardholme STW (μg/L)

Experimental conditions (Autotrace)- 600 mL final effluent samples, with the addition of 400 mL of methanol, were loaded on C_8 1 g Isolute cartridges at 10 mL/min. The cartridges were dried with nitrogen (1 hr) and eluted with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).

Native AE in effluent (μg/L)						
	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	0.187	<0.04	0.216	<0.04	0.188	1.531
EO_1	0.053	<0.04	0.045	0.108	<0.04	<0.04
EO_2	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_3	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_4	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_5	<0.04	0.059	<0.04	<0.04	<0.04	<0.04
EO_6	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_7	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_8	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_9	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{10}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{11}	<0.04	<0.04	Int	<0.04	<0.04	<0.04
EO_{12}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{13}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{14}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{15}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{16}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{17}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{18}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{19}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
EO_{20}	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04
Totals	<1.000	<0.859	<0.981	<0.908	<0.988	<2.331
Total	**<7.068**					

Int = isobaric interference

7.6 Confirmation of MDL with individual EOs

For a more thorough determination of MDL, individual EOs were spiked into final effluent at concentrations just above the estimated MDL of 0.02 μg/L (Section 7.5).

The EOs listed in Table 7.14 were selected, as these compounds were not present in the blank final effluent. Effluent from Broardholme STW was filtered through a GF/C filter to a total volume of approximately 4 L. Then 6 aliquots of 600 mL were measured into 1 L bottles. Four of the six effluent samples were spiked with 100 μL of the 1μM AE mix in methanol (Section 5.1.4). Two of the six effluent samples were analysed as blanks. The spiked samples contained individual EOs at concentrations as listed in Table 7.14. A volume of 400 mL of methanol (to give a final concentration of 40 % v/v) was then added to each bottle before application of the SPE procedure (Section 5.3.1).

Table 7.14. Concentration of individual EOs spiked into final effluent

Ethoxylate	**MW**	**Concentration in 1 μM (μg/mL)**	**Concentration in effluent (μg/L)**
$C_{12}EO_8$	538.8	0.539	0.090
$C_{14}EO_8$	566.8	0.567	0.095
$C_{16}EO_8$	594.9	0.595	0.099
$C_{18}EO_8$	622.9	0.623	0.104

The extracts were derivatised and analysed by LC/MS according to procedures in Sections 5.4 and 5.5.1., respectively. The LC/MS was calibrated over the range 0, 50, 100 and 200 nM of the derivatised EO mix. The results of the measured concentrations of $C_{12\text{-}18}EO_8$ are shown in Table 7.15.

Table 7.15. Measured recoveries of individual EOs spiked into final effluent.
Mean concentration (n = 4)

Experimental conditions (Autotrace)- 600 mL final effluent samples (spiked and unspiked), with the addition of 400 mL of methanol, were loaded on C_8 1 g Isolute cartridges at 10 mL/min. The cartridges were dried with nitrogen (1 hr) and eluted with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).

EO	Measured concentration (µg/L)	sd	MDL (µg/L)
$C_{12}EO_8$	0.079	0.006	0.021
$C_{14}EO_8$	0.101	0.005	0.017
$C_{16}EO_8$	0.082	0.008	0.025
$C_{18}EO_8$	0.087	0.012	0.039

The results for the MDL in Table 7.15 were calculated according to equation [7.1]. No signal for the $C_{12\text{-}18}EO_8$ in the two blank determinations was detected.

$$MDL = t \times sd \qquad [7.1]$$

Where t is the student's t value for 3 degrees of freedom at 95 % confidence (= 3.182).

The results calculated by this method are in good agreement with the estimated detection limit of 0.02 µg/L for each EO used in Section 7.5. The definition of MDL as used in this work is for the full analytical procedure.

7.7 Reproducibility of the SPE LC/MS procedure

Data quoted in Section 7.5 for effluent samples (Table 7.10) was reproduced on a different day to give an indication of the robustness of the methodology. Applying the t-test to the means of data in Table 7.16 and Table 7.10, e.g for C_{16} $EO_{0\text{-}4}$ and C_{18} $EO_{0\text{-}4}$, showed that statistically, with a 95 % confidence, the recovery data in the former was better. However it is considered that in environmental data of this type, where the effluent can differ from sample to sample, both sets of data are acceptable in terms of recovery performance. The data in Table 7.16 still shows overall mean recovery of the EOs analysed greater than 80 %.

Table 7.16. Recovery of 100 μg/L of total AE spiked into final effluent-reproducibility data.

Values quoted are mean (%RSD), (n = 3)

Experimental conditions (Autotrace)- 600 mL final effluent samples (spiked and unspiked), with the addition of 400 mL of methanol, were loaded on C_8 1 g Isolute cartridges at 10 mL/min. The cartridges were dried with nitrogen (1 hr) and eluted with 15 mL of methanol/DCM/MTBE (2/1/1, v/v).

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	51 (18)	58 (10)	53 (35)	74 (10)	71 (16)	53 (29)
EO_1	65 (7)	80 (5)	93 (13)	92 (12)	90 (14)	86 (11)
EO_2	84 (18)	85 (4)	83 (11)	95 (3)	89 (9)	83 (9)
EO_3	82 (7)	89 (5)	98 (6)	87 (7)	88 (2)	94 (11)
EO_4	80 (6)	88 (5)	93 (12)	88 (5)	90 (6)	84 (5)
Mean EO_{0-4}	**73 (11)**	**80 (6)**	**84 (15)**	**87 (8)**	**86 (9)**	**80 (13)**
EO_5	83 (12)	90 (1)	92 (6)	81 (7)	89 (11)	70 (6)
EO_6	80 (14)	87 (10)	87 (12)	85 (10)	84 (6)	65 (21)
EO_7	84 (8)	89 (7)	87 (4)	82 (5)	84 (11)	68 (22)
EO_8	84 (8)	86 (3)	86 (3)	85 (9)	84 (11)	67 (12)
EO_9	86 (3)	84 (3)	86 (9)	89 (11)	89 (6)	70 (18)
EO_{10}	89 (6)	88 (6)	86 (3)	87 (13)	90 (5)	71 (20)
EO_{11}	88 (2)	87 (3)	85 (4)	87 (14)	91 (2)	77 (17)
EO_{12}	89 (4)	89 (0)	89 (5)	91 (10)	92 (1)	79 (10)
EO_{13}	90 (4)	85 (3)	89 (8)	94 (16)	90 (3)	81 (9)
EO_{14}	84 (0)	91 (7)	86 (4)	87 (8)	91 (8)	81 (9)
EO_{15}	90 (3)	83 (4)	85 (4)	91 (15)	87 (6)	81 (8)
EO_{16}	84 (5)	78 (10)	83 (5)	80 (9)	87 (8)	84 (8)
EO_{17}	87 (1)	80 (2)	86 (9)	82 (12)	86 (8)	82 (4)
EO_{18}	85 (7)	86 (8)	82 (11)	86 (4)	88 (9)	81 (4)
EO_{19}	92 (8)	86 (4)	86 (10)	84 (3)	92 (3)	81 (9)
EO_{20}	88 (6)	87 (20)	90 (11)	77 (10)	91 (6)	80 (8)
Mean EO_{5-20}	**86 (6)**	**86 (6)**	**87 (7)**	**86 (10)**	**88 (7)**	**76 (12)**

7.8 Conclusion

Initial work showed that a simple one solvent elution system using methanol was ineffective in AE recovery. It was also noticed that if methanol was not added to the aqueous sample significant losses of hydrophobic components ($C_{18}EO_0$-EO_{10}) occurred, an effect that was more apparent at lower concentrations of AEs. Experiments with different mixed elution solvents e.g. combinations of methanol: MTBE: DCM, improved recoveries, but problems were still witnessed with $C_{18}EO_0$-EO_{10}. It was shown that the poor recovery of these ethoxymers could be attributed to retention on glassware and the flow tubing of the Autotrace SPE system. Losses were minimised by the addition of 40 % (v/v) methanol to the sample prior to loading, without any adverse effect being observed on the recovery of the polar ethoxylate species (e.g. $C_{12}EO_{10}$-EO_{20}).

An automated SPE step has been developed to allow both pre-concentration and clean-up of the environmental samples. The method provides more efficient recovery of AEs across the C_{12} to C_{18} range than previously reported in the literature. Recoveries from final effluent spiked at 100 μg/L total AE, for the 126 species analysed, were found to be in the range 55 – 117%, with approximately 100 of the individual analytes having recoveries of 90 –105 %. An LOD of 0.02 μg/L for individual ethoxylate components was reported with the instrument operated in scan mode over the range m/z 300 to 1300. The method was applied to sewage effluent and influent samples, with AEs determined at approximately 7 and 5000 μg/L respectively. A paper describing the analysis of sewage influent and effluent by SPE in combination with phthalic anhydride derivatisation and LC/MS has just been published [9].

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METHOD DEVELOPMENT AND ANALYSIS OF SEDIMENT AND SEWAGE SLUDGE SAMPLES

8.1 Introduction

Surfactants are discharged into the sewage system where removal is either by biodegradation or adsorption onto sewage sludge. For risk assessment purposes, the eventual discharge of the sludge must be considered. This may be in effluents to a receiving river, (such that it ends up in sediment), or it may be used on land as a fertiliser. Hence screening methods for the analysis of surfactants in sludge, agricultural soil and sediment are required. For a literature review of work done in this area, with particular reference to alcohol ethoxylates (AEs), refer to Chapter 3.

The aim of the work described in this chapter is to develop and compare extraction methodologies for sediments and sludges using techniques such as Soxhlet, ultrasonic and accelerated solvent extraction (ASE) with subsequent analysis by methods already developed in Chapter 6 (derivatisation and liquid chromatography/mass spectrometry (LC/MS)) and Chapter 7 (solid phase extraction (SPE)). Methodology describing extraction of AEs from solid samples is available in the literature (Section 3.3.2), but the detailed recovery of AEs in the range $C_{12-18}EO_{0-20}$ (126 ethoxymers) has not been reported before. The approach used for sewage influents and effluents, where AE recovery was optimised over the entire range, will be applied to the solid samples.

In the method development that follows a mix of commercially available individual ethoxylates (EOs) will be used to reduce and simplify data processing of extracted ions in analysis by LC/MS. The method developed will then be applied to the full commercial mix of AEs

8.2 Soxhlet and ultrasonic extraction of individual EOs from river sediment

8.2.1 SAMPLE COLLECTION AND PRETREATMENT

Samples of river sediment, which had previously been preserved with formaldehyde, were oven-dried at 80 °C and graded according to particle size using a Fritsch Analysette 3 pro sieve shaker (Idar-Oberstein, Germany). Sediment particle sizes of 75 – 250 μm were then stored frozen at -20°C until required for method development.

8.2.2 EXTRACTION PROCEDURES

Buchi Soxhlet

Soxhlet extraction was carried out on a Buchi B-810 Soxhlet extraction apparatus from Fisher Scientific (Loughborough, UK). In its design and use this apparatus can be compared to traditional Soxhlet (Figure 3.6). Solvent condenses and drips back through the sample in the thimble with some heat being transferred to the sample. Whatman cellulose extraction thimbles (Maidstone, UK) were pre-washed on the unit before loading with 10 g of sediment. The methodology consisted of extraction with 110 mL of solvent (e.g. methanol) for 6 or 16 hr at 150 °C. Extracts were then treated in two different ways: being either concentrated to 10 mL using a Zymark Turbovap (Runcorn, UK) with a 1 mL aliquot being evaporated and derivatised (Section 5.4.1) or the extract was made up to 200 mL in methanol, 300 mL of Ultrapure water added and the sample taken through the SPE procedure (Section 5.3.1) before being derivatised.

For the spiking of the sediment samples 1 mL of a 1 μM EO mix (see Section 5.1.4) in acetone was added to 10 g of sediment. In all cases this was then left in contact with the sediment overnight before extraction to allow some ageing to occur.

Ultrasonic extraction

AEs were also extracted from sediment by sonication with methanol at 50°C. Three 10 min extractions (50 mL and 2 x 40 mL) were carried out with the sediment separated from the extract by means of a centrifugation step. The combined extract was either concentrated to 10 mL or taken through an SPE clean up procedure. Details of treatment of these extracts are as described in Section 8.2.2.

8.2.3 *LC/MS ANALYSIS*

LC/MS was carried out according to procedures in Section 5.5.1. For the work with the individual EOs the following approach regarding calibration was used for two final extract concentrations:

Calibration procedure 1: For the extract, 1 mL of a 1 μM acetone EO spike was extracted into a final volume of 10 mL (equivalent to 100 nM EO). Therefore a calibration of 0, 50, 100 and 250 nM of each EO as a derivative was prepared from the 1 μM EO pyridine solution (Section 5.1.4).

Calibration procedure 2: For the extract, 1 mL of a 1 μM acetone EO spike was extracted into a final volume of 1 mL after SPE clean up (equivalent to 1 μM EO). A calibration of 0, 250, 500, 1000 and 1500 nM of each EO as a derivative was prepared.

Components used in the EO mix and concentrations are shown in Table 8.1. Individual stocks were prepared in methanol at nominal concentrations of 1000 μg/mL. These were then combined and diluted by adding a nominal 18.6 to 56.7 μL for $C_{12}EO_0$ to $C_{18}EO_8$, respectively to a 100 mL volumetric and making up to volume in acetone or pyridine.

Table 8.1 Concentrations of individual EOs in a 1 μM solution

Ethoxylate	MW	Concentration (μg/mL)	*Concentration in sediment (μg/kg)
$C_{12}EO_0$	186.3	0.186	18.6
$C_{14}EO_0$	214.4	0.214	21.4
$C_{16}EO_0$	242.4	0.242	24.2
$C_{18}EO_0$	270.5	0.271	27.1
$C_{12}EO_2$	274.4	0.274	27.4
$C_{14}EO_2$	302.5	0.303	30.3
$C_{16}EO_2$	330.5	0.331	33.1
$C_{12}EO_8$	538.8	0.539	53.9
$C_{14}EO_8$	566.8	0.567	56.7
$C_{16}EO_8$	594.9	0.595	59.5
$C_{18}EO_8$	622.9	0.623	62.3

(MW molecular weight)

* 1 mL spiked in 10 g sediment

8.2.4 *RESULTS AND DISCUSSION*

Buchi Soxhlet and ultrasonic comparison with no SPE clean up

Methanol is widely used in the literature for Soxtec (automated Soxhlet) [1] and ultrasonic [2] extraction of AE compounds from sludge/sediments. Results of an experiment comparing the use of methanol using Soxhlet and ultrasonic extraction are presented in Table 8.2. Applying the t-test to compare the means of the two techniques suggested no statistical difference at the 95 % confidence level for the recovery of AEs in the range EO_2- EO_8. The interference shown in the Soxhlet data was due to the presence of free alcohols in blanks and was traced back to the apparatus itself. A significant trend was the difference between the results obtained for EO_2 ethoxymers compared to those for EO_8 (e.g Soxhlet recoveries were obtained ranging from 95 -122 for EO_2 and 66 – 81 for EO_8). Lower recoveries of the more ethoxylated AEs were obtained; however this will become more apparent in the analysis of Commercial AEs (Section 8.3).

Table 8.2 Comparison of Buchi Soxhlet and ultrasonic extraction using methanol without clean-up

Values quoted are mean % recovery (% RSD), n = 3.

Experimental conditions- 10 g of sediment (spiked and unspiked) were extracted as follows: Soxhlet:- 110 mL of methanol for 6 hr on a Buchi Soxhlet. Ultrasonic:- methanol at 50 °C for 10 min, with 50 mL and 2 x 40 mL extracts combined. Final volume 10 mL, 1 mL evaporated and derivatised.

ETHOXYLATE	CONC. (µg/kg)	SOXHLET (6 HR)	ULTRASONIC
$C_{12}EO_0$	18.6	Int	112 (3)
$C_{14}EO_0$	21.4	Int	89 (4)
$C_{16}EO_0$	24.2	Int	73 (4)
$C_{18}EO_0$	27.1	Int	63 (5)
$C_{12}EO_2$	27.4	122 (28)	91 (8)
$C_{14}EO_2$	30.3	103 (12)	100 (16)
$C_{16}EO_2$	33.1	95 (10)	103 (9)
$C_{12}EO_8$	53.9	74 (12)	66 (15)
$C_{14}EO_8$	56.7	80 (7)	71 (13)
$C_{16}EO_8$	59.5	81 (9)	71 (11)
$C_{18}EO_8$	62.3	66 (7)	53 (11)
Mean $EO_2 - EO_8$		**89 (12)**	**79 (12)**

Int = Interference on Soxhlet apparatus

Soxhlet extraction with SPE clean-up using different solvents and times

The Soxhlet data shown in Table 8.3 was after the application of the SPE clean-up procedure. This allows a more concentrated extract to be obtained i.e. 1 mL final volume compared to a 10 mL final volume for the results in Table 8.2. This was mainly due to the elimination of co-extracted water and cellulose fibres which made concentration of the non-SPE cleaned extract to volumes lower than 10 mL difficult. In terms of LC/MS analysis, matrix interference in the two processes was shown to be similar. The data in Table 8.3 shows comparable recoveries for the 6 hr methanol data with that obtained in Table 8.2. The mean % recovery of EO_{2-8} (%, RSD) was 78 (4) with SPE, compared to 89 (12) without clean-up.

Table 8.3 Buchi Soxhlet extraction followed by SPE clean-up

Values quoted are mean % recovery (% RSD), n = 3.

Experimental conditions- 10 g of sediment (spiked and unspiked) were extracted by Soxhlet and cleaned up using SPE on a C_8 1 g Isolute cartridge (final volume after derivatisation, 1 mL)

ETHOXYLATE	CONC. (µg/kg)	METHANOL (6 HR)	METHANOL (16HR)	METHANOL/DCM (70/30, V/V) (6 HR)
$C_{12}EO_0$	18.6	Int	Int	Int
$C_{14}EO_0$	21.4	Int	Int	Int
$C_{16}EO_0$	24.2	Int	Int	Int
$C_{18}EO_0$	27.1	Int	Int	Int
$C_{12}EO_2$	27.4	85 (4)	83 (12)	83 (1)
$C_{14}EO_2$	30.3	112 (1)	92 (17)	84 (7)
$C_{16}EO_2$	33.1	94 (5)	84 (4)	71 (9)
$C_{12}EO_8$	53.9	69 (3)	75 (7)	68 (4)
$C_{14}EO_8$	56.7	74 (2)	72 (6)	71 (4)
$C_{16}EO_8$	59.5	62 (6)	68 (7)	60 (7)
$C_{18}EO_8$	62.3	54 (5)	53 (4)	53 (5)
Mean $EO_2 - EO_8$		**78 (4)**	**75 (8)**	**70 (5)**

Int = Interference on Soxhlet apparatus

Applying the t-test to compare the means of a methanol 6 hr extraction with and without SPE clean-up, suggested no statistical difference at the 95 % confidence level between the two techniques. However comparing the individual EO recovery for the 6 hr Soxhlet, a recovery of 85 and 122 % was obtained for $C_{12}EO_2$ with and without SPE, respectively. Similarly a recovery of 62 and 81 % was obtained for $C_{16}EO_8$ with and without SPE, respectively. Although these results had higher RSDs associated with them, the data without clean-up gave some better recoveries. However an increase in injection volume from 10 to 25 µL was required to detect the AEs in extracts concentrated to a final volume of 10 mL (no SPE clean-up). On a day to day basis the instrument performance would not meet the detection of such samples. A more robust method was provided by the more concentrated SPE extract.

Other data presented in Table 8.3 shows that increasing the time of extraction to 16 hr using methanol does not give improved recovery, for the ethoxylates monitored. The mean recovery of EO_{2-8} (%, RSD) for 6 and 16 hr Soxhlet being 78 (4) and 75 (8), respectively. The use of methanol/dichloromethane (DCM) caused solvent bumping problems on the Soxhlet apparatus, despite the addition of anti-bumping granules. The recoveries obtained with this solvent mixture did not show any improvement in comparison with methanol. A mean recovery of EO_{2-8} (%, (%RSD)) of 70 (5) with DCM was obtained compared to 78 (4) with methanol alone. DCM was included in the mixture as it was shown to be selective towards elution of the more hydrophobic AEs in reversed-phase SPE method development (Chapter 7). In Table 8.3 it is noted that the recovery of $C_{18}EO_8$ was not affected by use of a combined solvent (methanol/DCM, 70/30, v/v).

8.3 Comparison of Buchi Soxhlet and ultrasonic extraction of a commercial AE mixture from river sediment

8.3.1 METHODS

A more comprehensive AE recovery from river sediment was investigated in this section using the commercial AE mixture detailed in Section 5.1.2 and 5.1.3. This comprised of AEs in the range $C_{12-18}EO_{0-20}$ and would investigate more completely any data trends emerging from Section 8.2 with the individual EOs.

Methanol was used for both ultrasonic and Soxhlet extraction. The Soxhlet extraction was carried out over 6 hr. Method conditions were those used in Section 8.2.2 for Buchi Soxhlet and ultrasonic. For each evaluation triplicate AE spikes in sediment and blank sediment were tested. AEs were spiked onto the sediment (1 mL of the acetone solution detailed in Section 5.1.3, equivalent to 60 μg of total AE) and left overnight to allow the solvent to evaporate and enable the AEs to interact with the matrix. Although there was not any significant difference between SPE and non-SPE data in Section 8.3, SPE was used to allow a more concentrated extract necessary to meet the LC/MS detection requirements in full scan mode (Section 5.5.1), for the levels of AE spiked. Calibration standards over the range of

0 to 120 μg/mL of total AE were prepared as in Section 5.1.3 and the extracted ions monitored for C_{12} and C_{18} AEs (Section 5.5.3).

8.3.2 RESULTS AND DISCUSSION

The data presented in Table 8.4 shows clearly the data can be best interpreted by dividing the table into two parts, EO_{1-14} and $EO_{>14}$. Firstly let us consider EO_{1-14} recoveries for C_{12} and C_{18} AEs by the two techniques, Soxhlet and ultrasonic extraction. Applying the t-test to the means showed that statistically, with a 95 % confidence, the Soxhlet extraction was a superior method to the ultrasonic for both C_{12} and C_{18} recovery. This fact is also clearly demonstrated by the figures in bold in Table 8.4 which show mean recoveries for Soxhlet of 78 and 77 % for C_{12} and C_{18}, respectively, whereas for ultrasonic the mean recoveries are 55 and 50 % for C_{12} and C_{18}, respectively.

If we now consider $EO_{>14}$ it is apparent that the methods for both Soxhlet and ultrasonic extraction are not efficient in the recovery of these EOs. This effect is consistent with data showing that longer ethoxy chains have a greater affinity for soil components [3] with the chain being specifically absorbed due to a hydrogen-bond mechanism [4].

8.4 Comparison of Soxhlet and ASE extraction of a commercial AE mixture from river sediment

8.4.1 METHODS

Methodology for the determination of AEs in river sediment by ASE and Soxhlet was compared. Previous work (Section 8.3) has shown that recoveries of the higher EOs (EO_{15-20}) are problematical, therefore extended Soxhlet times will be used. Extractions on the ASE will be compared to traditional Soxhlet extraction, with individual Soxhlet glassware used in preference to the Buchi Soxhlet due to previous blank problems. These two Soxhlet techniques were thought to be comparable in the mode of action.

Table 8.4. Comparison of commercial AE recovery, spiked at 6 mg/kg in sediment, using Soxhlet and ultrasonic extraction
Values quoted are mean % recovery (% RSD), n = 3.

Experimental conditions- 10 g of sediment (spiked and unspiked) were extracted as follows: Soxhlet:- 110 mL of methanol for 6 hr on a Buchi Soxhlet. Ultrasonic:- methanol at 50 °C for 10 min, with 50 mL and 2 x 40 mL extracts combined. SPE clean-up using a C_8 1 g Isolute cartridge (final volume after derivatisation, 1 mL)

	SOXHLET		ULTRASONIC	
	C_{12}	C_{18}	C_{12}	C_{18}
EO_0	Int	Int	67 (4)	79 (3)
EO_1	102 (12)	68 (7)	82 (6)	69 (3)
EO_2	78 (8)	64 (1)	75 (8)	56 (4)
EO_3	76 (9)	75 (16)	78 (3)	56 (5)
EO_4	67 (8)	74 (9)	69 (7)	57 (2)
EO_5	73 (3)	86 (16)	66 (5)	74 (9)
EO_6	69 (2)	71 (5)	61 (2)	54 (7)
EO_7	67 (6)	67 (4)	65 (3)	46 (5)
EO_8	73 (5)	75 (2)	63 (5)	52 (10)
EO_9	73 (4)	77 (4)	60 (3)	54 (5)
EO_{10}	75 (3)	76 (2)	55 (3)	50 (6)
EO_{11}	96 (2)	83 (5)	41 (5)	45 (6)
EO_{12}	83 (11)	84 (3)	28 (6)	38 (6)
EO_{13}	116 (6)	89 (1)	19 (2)	28 (6)
EO_{14}	48 (25)	83 (4)	10 (7)	18 (9)
Mean EO_{1-14}	**78 (8)**	**77 (6)**	**55 (5)**	**50 (6)**
EO_{15}	18 (59)	74 (8)	6 (13)	12 (5)
EO_{16}	nd	45 (24)	nd	5 (88)
EO_{17}	nd	22 (34)	nd	2 (88)
EO_{18}	nd	nd	nd	nd
EO_{19}	nd	nd	nd	nd
EO_{20}	nd	nd	nd	nd

Int = Interference

nd = not detected

The following sediment spikes were prepared:

High spike Sediment, 60 mg/kg of AE (600 μg AE/ 10 g sediment): prepared by adding 7 mL of 600 μg/mL AE in acetone spike (Section 5.1.3) to 70 g of sediment, allowing the solvent to evaporate and mixing thoroughly. This was left to contact the sediment for four days before analysis.

Low spike Sediment, 6 mg/kg of AE (60 μg AE/ 10 g sediment): prepared by adding 7 mL of a 60 μg/mL AE in acetone spike (Section 5.1.3) to 70 g of sediment, allowing the solvent to evaporate and mixing thoroughly. This was left to contact the sediment for four days before analysis.

The Soxhlet extraction was carried out over 16 hr using methanol and cellulose extraction thimbles from Schleicher and Schuell supplied by VWR (Lutterworth, UK). Spiked sediment, as prepared above, and blank sediment were analysed in triplicate 10 g aliquots. The final extract was concentrated on the turbovap to 10 mL. For high spike samples, 1 mL was concentrated to dryness under nitrogen. Low spiked samples were cleaned up through SPE (Section 5.3.1) to allow a more concentrated extract to be obtained to meet the LC/MS detection requirements in full scan mode (Section 5.5.1) for the levels of AE spiked. Both sample types were then derivatised according to procedures in Section 5.4.1. Calibration standards over the range of 0 to 120 μg/mL of total AE were prepared as in Section 5.1.3. ASE extraction was carried out on an ASE 200 from Dionex Ltd. (Camberley, UK). The following conditions were used: temperature 150 °C, pressure 1500 psi, preheat 7 min, static 5 min, flush volume 60 % (22 mL cell), purge time 180 s. The cell was extracted twice using methanol with the extracts combined in a turbovap tube. The same approach as for Soxhlet was carried out for the cleanup, concentration, derivatisation and LC/MS analysis of extracts.

8.4.2 RESULTS AND DISCUSSION

For sediment spiked at 60 mg/kg of total AE, a comparison of data in Table 8.5 and 8.6 shows that the ASE was much more efficient at recovering the higher EOs. This becomes most apparent at around EO_{18}, where recovery for C_{12}, C_{13}, C_{14}, C_{15}, C_{16} and C_{18} was 27, 28, 45, 37, 48 and 66 % for Soxhlet and 91, 55, 82, 53, 78 and

100 % for ASE, respectively. However both methods performed well for EO_{0-17} ethoxylates, with similar mean recoveries at 60 mg/kg AE using both techniques.

A similar comparison of Soxhlet and ASE recovery for sediment spiked at 6 mg/kg of total AE can be made from data shown in Table 8.7 and 8.8, respectively. At the lower levels of individual EOs, contained within the AEs spiked at the lower concentration, it is evident from the data that EO_0 recovery is not quite as good. In Table 8.8, for example, C_{16} and C_{18} EO_0 recovery is 184 and 208 %, respectively indicating the normal approach of subtracting amounts in the blank sediment did not work. These results are attributed to the high native free alcohols present in the sediment, where the data using ASE extraction recovered native levels of 0.098 and 0.562 mg/kg, respectively, for these two alcohols (Table 8.9). For this reason the mean of EO_{1-17} has been compared for the two techniques. Applying the t-test to these results suggested no statistical difference at the 95 % confidence level between the two techniques. However on consideration of EO_{18-20} data in Table 8.7 and 8.8 there is clear evidence of the superiority of the ASE technique for these ethoxylates. Data showing the amount of individual EOs present in the 6 mg/kg AE spike is also shown in Table 8.10. The limits of detection (LODs) of the target compounds in the sediment were calculated by a signal to noise ratio of three (the ratio between the intensity of the signal of selected AE and the noise in the extracted ion chromatogram. In Table 8.10 certain EOs were considered to be present at the LOD e.g. $C_{14}EO_{1-3}$, which was then estimated to be 0.01 mg/kg for each component. This LOD or method detection limit (MDL) was assessed for 10 g of sediment extracted to a final volume of 1 mL, via an SPE clean-up, and should be adjusted according to the weight of sample and final volume applied, in future assessment of LOD.

The data presented in Section 8.4 generally shows that both ASE and Soxhlet can be applied effectively to extract AE from sediment samples. A Soxhlet time of 16 hr with methanol extraction appeared essential to improve recovery of the high EOs. A comparison with the 6 hr Soxhlet data obtained in Section 8.3 clearly demonstrates this. In Table 8.4, $C_{12}EO_{14-16}$ were recovered at 48 %, 18 % and undetectable in 6 hr Soxhlet data, whereas Table 8.7 shows recovery of 70 %, 56 % and 40 %, respectively for the same EOs, subjected to a 16 hr procedure. However the ASE demonstrated more effective recovery over the entire ethoxylate range EO_{0-20}, with data for EO_{18-20} superior to 16 hr Soxhlet data. In the data shown in Table 8.9 there was also evidence that the ASE may be more efficient in the extraction of native alcohols present. For example 0.562 mg/kg of $C_{18}EO_0$ was extracted by ASE, compared to 0.253 mg/kg extracted by Soxhlet from the sediment used in the method development.

Table 8.5 AE recovery from a sediment spiked at 60 mg/kg using 16 hr Soxhlet extraction with methanol, no SPE clean-up. The extract was transferred to a Turbovap tube, concentrated to a final volume of 10 mL, with 1 mL taken to dryness and derivatised.
Values quoted are mean % recovery (% RSD), n = 3.

	C_{12}		C_{13}		C_{14}		C_{15}		C_{16}		C_{18}	
EO_0	54	(15)	49	(9)	120	(9)	89	(4)	89	(10)	88	(15)
EO_1	96	(6)	106	(6)	139	(15)	79	(11)	103	(14)	84	(2)
EO_2	103	(8)	115	(9)	117	(13)	117	(10)	115	(18)	90	(6)
EO_3	91	(10)	105	(3)	97	(10)	99	(6)	91	(10)	82	(3)
EO_4	89	(6)	94	(8)	86	(19)	88	(4)	80	(9)	82	(2)
EO_5	85	(5)	98	(9)	91	(15)	97	(8)	87	(11)	86	(10)
EO_6	99	(7)	99	(1)	95	(1)	96	(4)	91	(1)	91	(5)
EO_7	93	(4)	87	(2)	86	(3)	90	(3)	91	(12)	95	(1)
EO_8	92	(6)	104	(7)	89	(8)	95	(6)	99	(4)	95	(4)
EO_9	97	(8)	92	(5)	89	(7)	101	(6)	92	(8)	98	(7)
EO_{10}	94	(7)	96	(10)	104	(3)	100	(2)	97	(2)	104	(3)
EO_{11}	92	(4)	101	(5)	86	(6)	101	(13)	105	(3)	106	(3)
EO_{12}	95	(5)	103	(11)	103	(5)	104	(12	94	(6)	102	(6)
EO_{13}	97	(1)	83	(14)	99	(9)	105	(21)	98	(13)	106	(5)
EO_{14}	82	(5)	90	(12)	92	(8)	73	(21)	100	(7)	103	(4)
EO_{15}	84	(21)	75	(17)	73	(5)	66	(13)	97	(3)	96	(6)
EO_{16}	65	(27)	67	(18)	66	(19)	63	(6)	78	(15)	93	(9)
EO_{17}	51	(30)	54	(12)	58	(38)	74	(26)	73	(20)	84	(11)
Mean EO_{0-17}	**87**	**(10)**	**90**	**(9)**	**94**	**(11)**	**91**	**(10)**	**93**	**(9)**	**93**	**(6)**
EO_{18}	27	(19)	28	(20)	45	(42)	37	(16)	48	(9)	66	(21)
EO_{19}	20	(12)	13	(88)	21	(46)	13	(173)	28	(41)	51	(22)
EO_{20}	nd	nd	nd	nd	nd	nd	nd	nd	16	(15)	27	(24)

nd = not detected

Table 8.6 AE recovery from a sediment spiked at 60 mg/kg using ASE extraction with methanol, no SPE clean-up

Values quoted are mean % recovery (% RSD), n = 3.

Experimental conditions- extractions were carried out on an ASE 200 using methanol at 150 °C and 1500 psi for 7 min (preheat), 5 min (static), flush volume 60 % (22 mL cell) and purge time 180 s. The extracts from 2 cycles were combined in a Turbovap tube, concentrated to a final volume of 10 mL, with 1 mL taken to dryness and derivatised.

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	78 (14)	69 (6)	131 (8)	90 (9)	99 (11)	99 (3)
EO_1	101 (3)	93 (2)	109 (14)	60 (8)	115 (5)	91 (10)
EO_2	101 (6)	107 (6)	100 (23)	107 (6)	96 (4)	98 (12)
EO_3	98 (13)	102 (6)	109 (11)	102 (16)	96 (11)	98 (5)
EO_4	92 (9)	103 (5)	94 (8)	89 (15)	86 (14)	101 (7)
EO_5	94 (5)	98 (5)	92 (10)	97 (10)	97 (10)	89 (8)
EO_6	95 (10)	96 (4)	95 (5)	91 (2)	96 (1)	93 (10)
EO_7	90 (1)	91 (4)	102 (13)	83 (10)	97 (5)	93 (1)
EO_8	90 (8)	104 (5)	84 (4)	83 (10)	93 (2)	95 (4)
EO_9	100 (4)	91 (6)	84 (4)	82 (13)	89 (5)	96 (9)
EO_{10}	95 (2)	97 (3)	102 (3)	102 (4)	109 (3)	96 (4)
EO_{11}	102 (4)	101 (4)	91 (8)	78 (8)	112 (5)	101 (2)
EO_{12}	94 (4)	101 (3)	95 (6)	83 (7)	101 (4)	98 (3)
EO_{13}	103 (3)	86 (8)	84 (6)	88 (8)	96 (2)	104 (9)
EO_{14}	93 (2)	81 (11)	98 (9)	91 (8)	98 (3)	102 (5)
EO_{15}	91 (3)	82 (3)	84 (9)	80 (19)	95 (4)	97 (10)
EO_{16}	89 (3)	84 (3)	84 (6)	81 (10)	95 (6)	92 (3)
EO_{17}	93 (6)	83 (11)	96 (9)	90 (25)	91 (11)	95 (4)
Mean EO_{0-17}	**94 (6)**	**93 (5)**	**96 (9)**	**88 (11)**	**98 (6)**	**97 (6)**
EO_{18}	91 (15)	55 (15)	82 (17)	53 (32)	78 (6)	100 (4)
EO_{19}	92 (1)	78 (10)	73 (11)	79 (30)	72 (7)	97 (6)
EO_{20}	76 (12)	62 (11)	76 (13)	80 (8)	93 (9)	96 (4)

Table 8.7 AE recovery from a sediment spiked at 6 mg/kg using Soxhlet extraction for 16 hr with methanol. The extract was cleaned up using a C_8 1 g Isolute cartridge.

Values quoted are mean % recovery (% RSD), n = 3.

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	95 (14)	44 (22)	158 (20)	103 (17)	143 (15)	63 (27)
EO_1	60 (18)	72 (12)	102 (26)	41 (4)	112 (29)	62 (9)
EO_2	73 (18)	72 (46)	135 (19)	45 (2)	107 (33)	73 (3)
EO_3	95 (12)	81 (9)	104 (20)	68 (5)	80 (18)	69 (12)
EO_4	73 (6)	75 (9)	75 (17)	72 (15)	74 (12)	66 (20)
EO_5	78 (14)	74 (5)	76 (16)	59 (7)	58 (7)	63 (15)
EO_6	76 (17)	75 (2)	68 (6)	66 (15)	67 (21)	66 (8)
EO_7	68 (5)	77 (8)	77 (15)	58 (3)	73 (11)	63 (13)
EO_8	69 (5)	74 (3)	63 (16)	69 (16)	68 (10)	66 (5)
EO_9	73 (5)	83 (9)	68 (6)	67 (4)	80 (9)	75 (3)
EO_{10}	72 (5)	76 (17)	72 (14)	70 (12)	70 (7)	75 (6)
EO_{11}	71 (11)	67 (10)	76 (5)	59 (8)	68 (9)	72 (4)
EO_{12}	68 (5)	71 (17)	70 (7)	70 (10)	78 (5)	73 (7)
EO_{13}	71 (9)	60 (10)	70 (12)	56 (6)	79 (13)	78 (11)
EO_{14}	70 (10)	51 (11)	65 (11)	60 (11)	81 (5)	75 (14)
EO_{15}	56 (15)	55 (6)	54 (19)	45 (26)	77 (14)	77 (18)
EO_{16}	40 (22)	45 (21)	45 (11)	52 (13)	66 (8)	68 (15)
EO_{17}	24 (41)	23 (7)	29 (19)	28 (38)	45 (18)	53 (24)
Mean EO_{1-17}	**67 (13)**	**67 (12)**	**73 (14)**	**58 (12)**	**75 (13)**	**69 (11)**
EO_{18}	12 (41)	13 (44)	15 (46)	14 (88)	28 (33)	37 (25)
EO_{19}	4 (33)	nd	nd	nd	16 (40)	23 (39)
EO_{20}	1 (173)	nd	nd	nd	6 (90)	11 (64)

nd = not detected

Table 8.8 AE recovery from a sediment spiked at 6 mg/kg using ASE extraction with methanol and SPE clean-up

Values quoted are mean % recovery (% RSD), n = 3.

Experimental conditions- extractions were carried out on an ASE 200 using methanol at 150 °C and 1500 psi for 7 min (preheat), 5 min (static), flush volume 60 % (22 mL cell) and purge time 180 s. The extracts from 2 cycles were cleaned up using a C_8 1 g Isolute cartridge.

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	120 (4)	55 (8)	169 (3)	119 (4)	184 (6)	208 (4)
EO_1	68 (3)	77 (2)	83 (4)	56 (5)	80 (10)	67 (5)
EO_2	78 (6)	94 (56)	122 (10)	54 (10)	96 (21)	62 (10)
EO_3	93 (29)	85 (5)	104 (6)	72 (7)	84 (17)	74 (9)
EO_4	68 (8)	72 (8)	76 (13)	67 (8)	60 (8)	66 (2)
EO_5	69 (2)	70 (3)	74 (4)	59 (13)	60 (10)	66 (6)
EO_6	77 (7)	71 (7)	69 (4)	64 (13)	63 (5)	69 (5)
EO_7	72 (6)	72 (2)	75 (8)	65 (6)	71 (15)	67 (6)
EO_8	63 (7)	73 (6)	68 (5)	69 (7)	71 (7)	71 (5)
EO_9	66 (7)	72 (2)	70 (21)	73 (13)	75 (2)	73 (3)
EO_{10}	66 (1)	75 (4)	66 (5)	74 (6)	71 (2)	67 (3)
EO_{11}	64 (6)	71 (8)	67 (4)	69 (13)	78 (2)	72 (4)
EO_{12}	71 (4)	68 (7)	61 (2)	59 (6)	78 (8)	72 (8)
EO_{13}	71 (3)	70 (20)	68 (14)	62 (6)	84 (5)	70 (2)
EO_{14}	74 (4)	62 (9)	83 (3)	59 (19)	66 (5)	82 (8)
EO_{15}	68 (5)	62 (4)	67 (5)	50 (30)	84 (3)	79 (5)
EO_{16}	65 (5)	57 (6)	64 (1)	60 (17)	83 (6)	69 (3)
EO_{17}	69 (7)	57 (15)	69 (10)	42 (6)	80 (6)	79 (8)
Mean EO_{1-17}	**71 (6)**	**71 (10)**	**76 (7)**	**62 (11)**	**76 (8)**	**71 (5)**
EO_{18}	63 (12)	53 (14)	55 (18)	52 (19)	80 (4)	84 (11)
EO_{19}	61 (3)	68 (25)	57 (21)	63 (16)	89 (0)	82 (6)
EO_{20}	53 (3)	49 (33)	51 (9)	60 (31)	78 (3)	80 (6)

Table 8.9. Native levels of alcohols in sediment (mg/kg)

Values quoted are mean (%RSD), n = 3.

Experimental conditions- extractions were carried as follows:

ASE 200:- (2 cycles) using methanol at 150 °C and 1500 psi for 7 min (preheat), 5 min (static), flush volume 60 % (22 mL cell) and purge time 180 s. Soxhlet:- extraction for 16 hr with methanol.

All extracts were cleaned up using a C_8 1 g Isolute cartridge.

	ASE	Soxhlet
$C_{12}EO_0$	0.049 (11)	0.049 (10)
$C_{13}EO_0$	0.040 (20)	0.039 (26)
$C_{14}EO_0$	0.068 (18)	0.060 (36)
$C_{15}EO_0$	0.147 (10)	0.100 (2)
$C_{16}EO_0$	0.098 (5)	0.078 (9)
$C_{18}EO_0$	0.562 (5)	0.253 (7)

Table 8.10. Theoretical concentrations (mg/kg) of individual ethoxylates contained in the 6 mg/kg AE spike

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	0.032	0.079	0.013	0.039	0.008	0.053
EO_1	0.023	0.060	0.010	0.027	0.019	0.035
EO_2	0.023	0.065	0.010	0.031	0.022	0.036
EO_3	0.035	0.084	0.011	0.038	0.025	0.037
EO_4	0.047	0.110	0.020	0.053	0.029	0.058
EO_5	0.052	0.130	0.024	0.057	0.037	0.068
EO_6	0.067	0.126	0.021	0.057	0.043	0.075
EO_7	0.078	0.127	0.028	0.053	0.057	0.084
EO_8	0.093	0.123	0.034	0.053	0.064	0.106
EO_9	0.103	0.111	0.036	0.046	0.071	0.112
EO_{10}	0.103	0.105	0.040	0.042	0.071	0.120
EO_{11}	0.108	0.089	0.036	0.030	0.078	0.121
EO_{12}	0.099	0.068	0.035	0.022	0.064	0.133
EO_{13}	0.077	0.050	0.027	0.019	0.065	0.124
EO_{14}	0.071	0.024	0.027	0.015	0.055	0.089
EO_{15}	0.061	0.026	0.016	0.008	0.048	0.087
EO_{16}	0.043	0.012	0.014	0.005	0.038	0.055
EO_{17}	0.028	0.002	0.011	0.003	0.029	0.044
EO_{18}	0.022	0.005	0.007	0.002	0.017	0.032
EO_{19}	0.013	0.004	0.004	0.000	0.014	0.023
EO_{20}	0.009	0.002	0.000	0.000	0.006	0.019
Totals	**1.187**	**1.400**	**0.422**	**0.600**	**0.858**	**1.509**
Total						**6.0**

8.5 ASE methodology applied to sludge samples

8.5.1 SAMPLE COLLECTION AND PRETREATMENT

Sewage sludge was collected from Broardholme sewage treatment plant (Wellingborough, UK). Immediately after receipt of the sample the excess water was decanted and the remaining water and sludge centrifuged into pellets (4000 rpm, 20 °C, 10 min). The extraction procedure described below was carried out within 3 hr of the sample being taken.

8.5.2 METHODS

Duplicate 5 g aliquots were weighed out and mixed with approximately 5 g of hydromatrix, supplied by Varian (Walton-on-Thames, UK). The samples were transferred to 33 mL ASE extraction cells and extracted by ASE according to the method developed in Section 8.4. The following deviations were made to the procedure to accommodate the larger sample size in the 33 mL cell: A flush volume of 10 % was used and triplicate extractions were combined in a Turbovap tube.

Spiked sludge:
1 g freeze dried sewage sludge was weighed in triplicate into separate beakers. Two of these were spiked with 1 mL of a 600 μg/mL AE spike in acetone (Section 5.1.3). The acetone was allowed to fully evaporate (2 hr) before adding 4 mL of water and mixing and drying with approximately 5 g of hydromatrix. The mixed sample was then transferred to a 33 mL ASE and extracted as described above.

All extracts were taken to a final volume of 20 mL on the Turbovap, with 10 mL of this cleaned up by SPE by adding 190 mL of methanol, 300 mL of Ultrapure water and taking through the SPE procedure (Section 5.3.1). Samples were then derivatised and analysed by LC/MS according to procedures in Section 5.4.1 and 5.5.1, respectively. Calibration standards over the range of 0 to 120 μg/mL of total AE were prepared as in Section 5.1.3.

8.5.3 RESULTS AND DISCUSSION

The results of the spiked recovery data are shown in Table 8.11. The data shows that good recovery of AE has been obtained using the ASE, from a wet sludge dried with hydromatrix. Recoveries of 77, 71, 72, 80, 81 and 82 % for C_{12}, C_{13}, C_{14}, C_{15}, C_{16} and C_{18} AE homologues, respectively were obtained. The poorer replication of recoveries seen in Table 8.11 for certain EOs was due to two factors. For $C_{12}EO_{0-2}$ this was due to native levels in the dried sludge used for spiking and blank correction (note this was a different sludge sample to that reported in Table 8.12). Whereas for the $C_{15}EO_{19-20}$ ethoxymers, for example, the poor replication was due to the lower level of EOs present in the Commercial mix.

Table 8.12 shows the native levels of AE analysed in the sludge. The moisture/ dried solids content of the prepared sludge pellets was also determined (9.6 % dried solids) and results are quoted on a dry weight basis. Extraction of the wet sample, absorbed onto hydromatrix, eliminated losses of volatile AE by drying and also enabled the sample to be analysed within 3 hr of being taken, ensuring losses due to biodegradation were minimised. The LOD of < 0.4 mg/kg quoted in Table 8.12 is derived from work done in Section 8.4 and based on the sample weight and final volume of the sludge extract.

It is evident from the data in Table 8.12 that of the 163 mg/kg of total AEs found in sewage sludge, 93 % w/w of this was accounted for by the $C_{12-18}EO_0$ species or free alcohol, often described as fatty alcohols. These figures are surprising given the relatively low proportions of alcohol present in commercial mixtures of surfactants (e.g. Table 6.8) and the work done in Chapter 9, which showed the absence of high levels of free alcohol formed in the biodegradation of AEs. The other species detected were in the range EO_{1-3} and typically at much lower levels than the fatty alcohols.

These results are in agreement with other workers compiling data for the risk assessment of AEs: A laboratory continuous activated sludge (CAS) study fed with AE suggested a lower amount of alcohol formed [5] compared to the higher percentage of alcohols found in results of monitoring studies in sewage effluents in

Europe and Canada [6]. This indicates that there may be alcohols present from non-AE sources in the environment. Further work is needed to elucidate the composition and origins of the alcohol fraction so that this data may be incorporated into the aquatic risk assessment of linear AEs [6].

Table 8.11 Recovery of AEs spiked in sewage sludge (600 µg AE/ 5 g wet material)
Values quoted as mean % (individual replicates), n = 2.

ASE 200:- 1 g sludge (spiked and unspiked) was wetted with water (4 mL) and mixed with 5 g of hydromatrix. It was extracted with methanol at 150 °C and 1500 psi for 7 min (preheat), 5 min (static), flush volume 10 % (33 mL cell) and purge time 180 s. The extracts from 3 cycles were combined in a Turbovap tube, concentrated to a final volume of 20 mL, with 10 mL cleaned up using a C_8 1 g Isolute cartridge, taken to dryness and derivatised.

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	81 (64, 99)	67 (67, 67)	nd	nd	nd	nd
EO_1	46 (29, 62)	91 (94, 88)	nd	nd	56 (62, 50)	64 (64, 64)
EO_2	100 (126, 74)	90 (92, 88)	59 (81, 37)	nd	71 (67, 76)	105 (104, 106)
EO_3	84 (89, 78)	84 (86, 82)	78 (80, 76)	90 (88, 91)	92 (96, 88)	89 (90, 88)
EO_4	70 (63, 77)	72 (68, 76)	79 (80, 79)	72 (74, 70)	80 (83, 78)	84 (92, 75)
EO_5	77 (75, 80	59 (63, 56)	64 (58, 70)	83 (84, 83)	85 (91, 79)	77 (77, 76)
EO_6	67 (65, 69)	54 (52, 56)	69 (70, 67)	71 (74, 67)	88 (85, 82)	86 (85, 87)
EO_7	72 (75, 68)	51 (55, 47)	80 (83, 78)	75 (77, 72)	73 (74, 73)	76 (74, 79)
EO_8	77 (79, 74)	55 (51, 58)	79 (80, 78)	84 (89, 80)	83 (86, 80)	78 (79, 77)
EO_9	74 (72, 77)	67 (69, 66)	83 (83, 82)	73 (74, 73)	86 (91, 81)	77 (78, 77)
EO_{10}	77 (77, 78)	74 (74, 75)	67 (66, 68)	79 (82, 76)	83 (88, 79)	78 (79, 78)
EO_{11}	83 (86, 81)	73 (77, 68)	61 (65, 67)	80 (81, 80)	76 (75, 78)	82 (80, 85)
EO_{12}	81 (81, 81)	66 (66, 66)	74 (78, 71)	78 (78, 78)	86 (88, 84)	82 (81, 83)
EO_{13}	74 (72, 77)	67 (73, 61)	67 (71, 63)	79 (91, 66)	85 (91, 79)	88 (86, 89)
EO_{14}	83 (83, 84)	69 (67, 71)	70 (67, 73)	91 (97, 85)	79 (84, 73)	81 (80, 82)
EO_{15}	78 (84, 72)	71 (72, 69)	88 (83, 93)	94 (93, 94)	83 (82, 83)	83 (86, 80)
EO_{16}	84 (84, 84)	82 (86, 77)	76 (75, 78)	64 (67, 61)	85 (87, 84)	86 (83, 89)
EO_{17}	78 (78, 79)	65 (61, 68)	70 (78, 62)	76 (71, 81)	82 (83, 80)	92 (95, 88)
EO_{18}	82 (83, 81)	75 (88, 63)	74 (81, 68)	79 (74, 85)	90 (93, 88)	88 (88, 87)
EO_{19}	75 (76, 74)	79 (75, 83)	65 (63, 68)	99 (122, 76)	83 (84, 82)	82 (81, 82)
EO_{20}	76 (76, 76)	69 (66, 72)	71 (73, 70)	71 (56, 86)	77 (80, 74)	72 (73, 71)
Mean	**77**	**71**	**72**	**80**	**81**	**82**

nd = no data, due to high native levels in sample

Table 8.12. Native levels of AEs in sewage sludge, determined by ASE extraction of a wet sample (mg/kg, dry weight).
Values quoted as mean (individual replicates)

ASE 200:- 5 g wet sludge was mixed with 5 g of hydromatrix and extracted with methanol at 150 °C and 1500 psi for 7 min (preheat), 5 min (static), flush volume 10 % (33 mL cell) and purge time 180 s. The extracts from 3 cycles were combined in a Turbovap tube, concentrated to a final volume of 20 mL, with 10 mL being cleaned up using a C_8 1 g Isolute SPE cartridge, taken to dryness and derivatised.

	C_{12}	C_{13}	C_{14}	C_{15}	C_{16}	C_{18}
EO_0	4.6 (5.3, 3.9)	0.8 (0.9, 0.7)	16 (18, 14)	17 (18, 15)	21 (21, 21)	95 (111, 79)
EO_1	< 0.4	2.1 (2.1, 2.0)	< 0.4	1.1 (1.2, 1.0)	2.6 (3.1, 2.1)	< 0.4
EO_2	< 0.4	1.2 (1.5, 0.9)	0.9 (0.9, 1.0)	< 0.4	1.1 (1.4, 0.8)	< 0.4
EO_3	< 0.4	< 0.4	0.6 (0.5, 0.6)	< 0.4	< 0.4	< 0.4
[a] $EO_{4\text{-}20}$	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4

Total free alcohol	**154**
Total EO	**9**
Total all species	**163**
% free alcohol	**94**

[a] = all components in the range measured at this detection limit

8.6 Conclusion

This chapter has demonstrated that the phthalic anhydride derivatisation and LC/MS method can be applied to extracts of sediment and sewage sludge samples. Although not essential, depending on the levels of AE in the samples, the SPE clean-up procedure, developed in Chapter 7, allows a method detection limit of 0.01 mg/kg for individual ethoxylates to be reached. In terms of extraction methodology, ASE was shown to be the superior technique due to its ability to efficiently extract the entire ethoxylate range present in the Commercial samples analysed. Even with 16 hr extraction times the Soxhlet was unable to recover $EO_{18\text{-}20}$ from spiked river sediment samples. Over the range $EO_{0\text{-}17}$ Soxhlet and ASE were comparable. Ultrasonication proved to be an even weaker extraction method, with inferior recoveries to Soxhlet being obtained. The objective of this chapter was to develop a method that would give speciated AE data in the range

$C_{12-18}EO_{0-20}$ for sediments and sludges: ASE extraction followed by derivatisation and LC/MS has clearly fulfilled this objective.

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SECTION A- BIODEGRADATION OF HIGHLY ETHOXYLATED ALCOHOL ETHOXYLATES IN SEALED VESSEL TESTS: DETERMINATION OF ALCOHOL ETHOXYLATE PROFILE

9.1 Introduction

Biodegradation studies, using the International Standards Organisation (ISO) 14593 CO_2 headspace method [1] or sealed vessel test, were carried out on 'highly' ethoxylated (>20 EO) alcohol ethoxylates (AEs). The test substance, as the sole source of carbon and energy, is added to a mineral salts medium inoculated with a mixed population of micro-organisms and incubated in sealed vessels under a headspace of air. Biodegradation or mineralisation to CO_2 is determined by measuring inorganic carbon (IC) levels compared with blanks. The extent of biodegradation is expressed as a percentage of the theoretical IC production based on the amount of test substance, as organic carbon, added.

The aim of this piece of work was to confirm the ultimate biodegradability of the highly ethoxylated AEs. The impact of differing alkyl chain structures i.e oxo-AE compared to highly branched AE on biodegradability was also investigated. The biodegradation studies were undertaken by other workers within Unilever, however in an attempt to understand mechanisms of biodegradation in the two AE types, chemical analysis of the sealed vessels was also performed. The AE profile was determined using solid phase extraction (SPE), followed by phthalic anhydride derivatisation and liquid chromatography mass spectrometry with electrospray ionisation (LC/ESI-MS). The derivatisation had previously been found to give a more accurate ethoxylate fingerprint and also enhance ionisation of the lower ethoxylates and free alcohol, which can be important in studying biodegradation mechanisms.

9.2 Experimental

9.2.1 CHEMICALS AND REAGENTS

See also Section 5.1.1.

Two commercial AEs were used. These were Lutensol AO30 and Lutensol TO20 which were supplied by BASF (Ludwigshafen, Germany). Lutensol AO30 consists of a mixture of C_{13} and C_{15} oxo AE (50 % linear, 29 % methyl and 20 % ≥ ethyl) with average EO of 23. Lutensol TO20 consists of a C_{13} branched alkyl chain (2 to 3 methyl branches per chain) with average EO of 18. Alkyl chain branching and average EO number were determined by NMR, Unilever Research, Vlaardingen. Stock solutions of each AE were prepared at 5000 μg/mL in methanol. The internal standard was n-hexadecyl-d_{33} alcohol prepared as described in Section 5.1.3.

9.2.2 BIODEGRADATION STUDIES

Biodegradation of the two tested AE mixtures was performed according to the ISO protocol [1]. Briefly, the test system comprised of a batch system (160 mL vials) containing mineral salts medium, bacterial inoculum (mixed liquor, homogenised and settled from Broadholme sewage treatment works (STW), Ditchford Lane, Wellingborough). The test substance was added at typically 10 mg/L carbon. The test vessels were incubated in the dark with gentle agitation for a period of 28 days. The vessels were analysed twice weekly for IC in both the headspace (~60 mL) and the liquid phase (~100 mL). The IC from a blank (i.e. vessel containing no test compound) is subtracted from the test to obtain IC released during biodegradation of the test compound. A reference compound, sodium benzoate, was also run in parallel to confirm the viability of the bacterial inoculum. Once the test vessel had been measured, as described above, it was made available for AE analysis.

9.2.3 EXTRACTION PROCEDURES

Samples were analysed on day 0, 1, 2, 3, 4, 5, 6, 7 and 9 of the study. Control samples, consisting of test media without addition of test compound, were also received on the same days for analysis. The samples were transferred to a 500 mL bottle, the sample container washed with 55 mL (this was increased to 66 mL in later work) of methanol and combined portions taken through the SPE procedure described in Section 5.3.1. Spikes prepared at 20 mg/L in test medium were used to monitor the performance of the method after taking through the same SPE procedure.

Calibrations standards of 0, 200, 500, 1000 and 2,000 μg/mL of lutensol TO20 and Lutensol AO30 were analysed to assess the linearity over the concentration range. These were prepared by adding 0, 0.04, 0.1, 0.2 and 0.4 mL of the methanol stocks into separate HPLC vial, taking to dryness under nitrogen and derivatising.

9.2.4 LC/MS ANALYSIS

LC/MS and syringe infusion MS was carried out as detailed in Section 5.2 and 5.5.1 with the following exception. The AE derivatives were separated by means of an Ace 5 C_{18} 15 cm x 2.1 mm i.d from Advanced Chromatography Technologies (Aberdeen, UK).

9.3 Results and Discussion

9.3.1 OPTIMISATION OF THE MS PROCEDURE

Alcohol ethoxylate response was optimised by syringe infusion ESI-MS of a concentrated Lutensol AO30 derivative (25 mg / 1 mL derivatisation reagent) diluted 50-fold with mobile phase (50 : 50, v/v, solvent A : solvent B, Section 5.5.1) and infused at 250 μL/min. A fragmentor ramp of 100 to 396 V over the mass range of interest, m/z 347 to 2109 was optimised by this method. A typical mass spectrum of Lutensol AO30 by syringe infusion is included in Figure 9.1. This shows negative ion [M-H]$^-$ formation of the derivative with ions ranging from $C_{13}EO_0$-EO_{40} clearly visible. The theoretical *m/z* values for these ions are listed in Table 9.1. The average EO number from NMR data was calculated as 23, which approximately matched the LC/MS profile shown. Another series of ions showed the Lutensol AO30 to be a mixture of C_{13} and C_{15} alkyl chains, in the approximate ratio of 2:1, respectively.

Figure 9.1. Syringe infusion of Lutensol AO30

Conditions- negative ion ESI-MS, of an approximately 0.5 mg/mL derivative in mobile phase (water/ACN/THF/ammonia, 500/450/50/0.1, v/v) using syringe infusion at 250 μL/min. A fragmentor ramp from 100 to 396 V for *m/z* 347 to 2109 was applied.

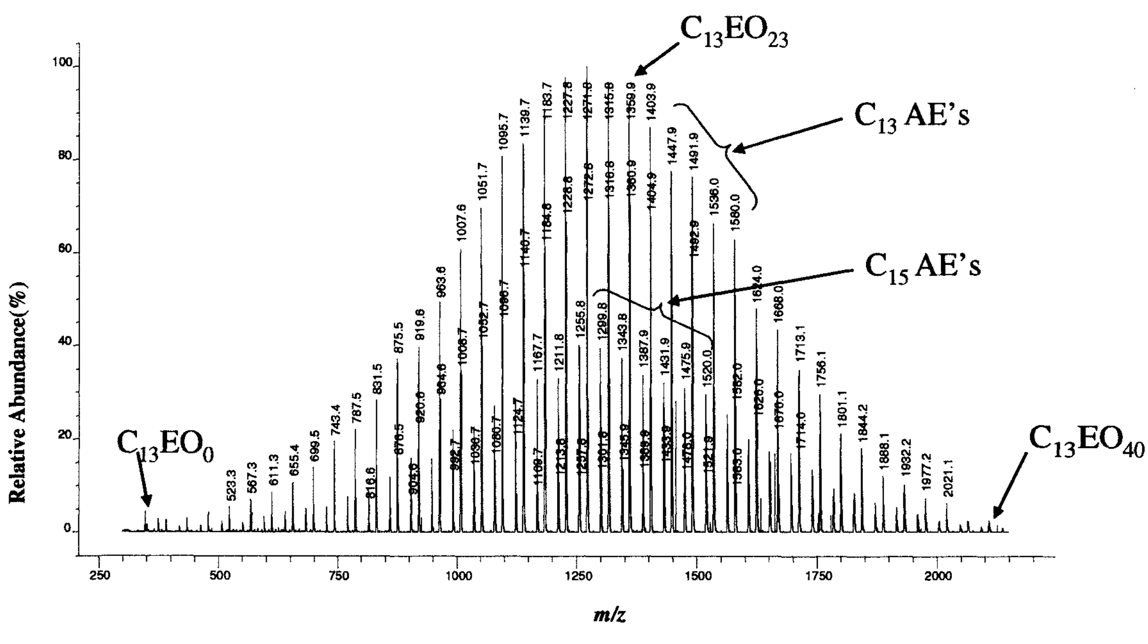


Table 9.1 Extracted ions used for the study of biodegradation of "parent" AE. The *m/z* values given are for the [M-H]$^-$ ions of the phthalic anhydride derivatives of each species.

Ethoxylate	C_{13} derivative *(m/z)*	Ethoxylate	C_{13} derivative *(m/z)*
EO0	347.2	EO21	1271.9
EO1	391.3	EO22	1315.9
EO2	435.3	EO23	1360.0
EO3	479.3	EO24	1404.0
EO4	523.4	EO25	1448.0
EO5	567.4	EO26	1492.1
EO6	611.4	EO27	1536.1
EO7	655.5	EO28	1580.1
EO8	699.5	EO29	1624.1
EO9	743.5	EO30	1668.2
EO10	787.6	EO31	1712.2
EO11	831.6	EO32	1756.2
EO12	875.6	EO33	1800.3
EO13	919.6	EO34	1844.3
EO14	963.7	EO35	1888.3
EO15	1007.7	EO36	1932.4
EO16	1051.7	EO37	1976.4
EO17	1095.8	EO38	2020.4
EO18	1139.8	EO39	2064.5
EO19	1183.8	EO40	2108.5
EO20	1227.9		

9.3.2 DATA ANALYSIS

Concentrations measured in the test vessels and results expressed as the % removal of "Parent" AE are presented in Table 9.2. These values were calculated by comparison of the total area under the C_{13} and C_{15} envelopes with that of a calibration standard. AE response was linear over the concentration range ($R^2 >$ 0.995), enabling AE in samples to be calculated by comparison of areas with those in a single point standard of 2000 μg/mL total AE. Total ion chromatograms (TICs) showing the data analysis described above are shown in Figure 9.2. Internal standard area was used to monitor derivatisation efficiency and also any injection error or ion suppression effects in the MS. Recovery data for spiked samples was as follows: Lutensol TO20 89.8 ± 3.1 % and Lutensol AO30 90.4 ± 3.2 % (n=3). Results in Table 9.2 were not corrected for SPE recovery. The measured concentrations for Lutensol TO20 and Lutensol AO30 at day 0 in the test vessel were 85.7 and 90.6 %, respectively of the known concentrations prepared in the study. The % removal of "parent material" quoted was calculated by comparison of the measured concentration on a particular day to the corresponding measured value on day 0.

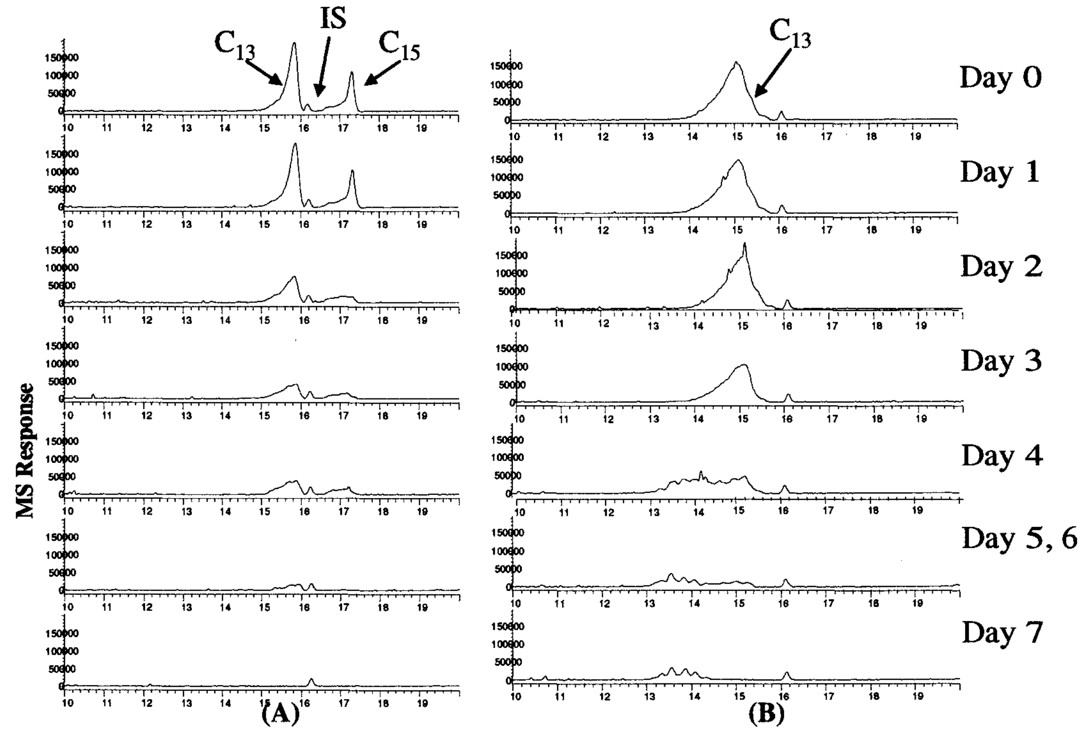
Table 9.2 Measured concentrations in test vessels (mg/L) with % removal of "parent material" in parentheses

	MEASURED CONCENTRATION (mg/L) (% REMOVAL OF "PARENT MATERIAL")	
Day of study	Lutensol AO30	Lutensol TO20
0	14.6 (0)	13.8 (0)
1	13.4 (7.8)	12.9 (6.0)
2	6.9 (52.8)	13.2 (4.2)
3	4.6 (68.2)	9.4 (31.8)
4	4.7 (67.4)	8.3 (39.5)
5	1.6 (89.1)	No data
6	No data	3.7 (73.1)
7	<0.2 (100)	2.5 (81.8)
9	<0.2 (100)	2.3 (83.2)

Sample results marked "no data" (Table 9.2) were due to error in the derivatisation process. The visual disappearance of parent material for Lutensol AO30 and Lutensol TO20 can clearly be seen in Figure 9.2. Extracted ion chromatograms (EICs) for the individual ethoxylate derivatives (Table 9.1) were also processed. The MS response for each ethoxylate was tabulated on selected days up to day 7 and by plotting the data AE profiles throughout the biodegradation process could be determined. Figure 9.3 shows the comparison of this data for Lutensol AO30 and Lutensol TO20 for the C_{13} AE series for each sample. There was clearly a difference seen in this data with a much more pronounced build up of the lower ethoxylates in Lutensol TO20 (Figure 9.3(A)). This is largely explained by the difference in structures of the two AE samples analysed. Lutensol AO30 is classed as a linear "oxo" type structure (Figure 9.4) whereas Lutensol TO20 contains a highly branched alkyl chain (Figure 9.5)

Figure 9.2 Total ion chromatograms of Lutensol AO30 (A) showing C_{13} and C_{15} alcohol ethoxylates and Lutensol TO20 (B) showing C_{13} alcohol ethoxylates.

Experimental conditions- negative ion ESI-MS of phthalic anhydride derivatives separated on an Ace 5 C_{18} 15 cm x 2.1 mm i.d. 5 μm column from Advanced Chromatography Technologies.



The biodegradation of Lutensol AO30 in a sealed vessel test was rapid with complete removal of "parent AE" wthin the first 7 days of the study. For Lutensol TO20 there was only 83.2 % removal of parent material in the first 9 days of the study with the residue comprising $C_{13}EO_{1-3}$ AE. The build up of the lower ethoxylates in the case of Lutensol TO20 required closer monitoring as these species have more toxicity in the environment [2]. Lutensol AO30 appeared to biodegrade much more readily without the appearance of a significant amount of these intermediates. One clear mechanism of biodegradation for the Lutensol TO20 appears to be non-oxidative loss of C_2 units from the hydrophile end resulting in a build up of lower ethoxylates described above. In the case of Lutensol AO30 there was only a slight build up of these species. Further work was necessary to confirm central fission as the main mechanism of biodegradation resulting in long chain polyethylene glycol (PEG) compounds. It was also necessary to assess the disappearance of PEG. Central fission is also proposed to give a free alcohol or

Figure 9.3. Biodegradation of Lutensol TO20 (A) displayed as the MS response of each C_{13} ethoxylate on selected days of analysis compared to that of Lutensol AO30 (B)

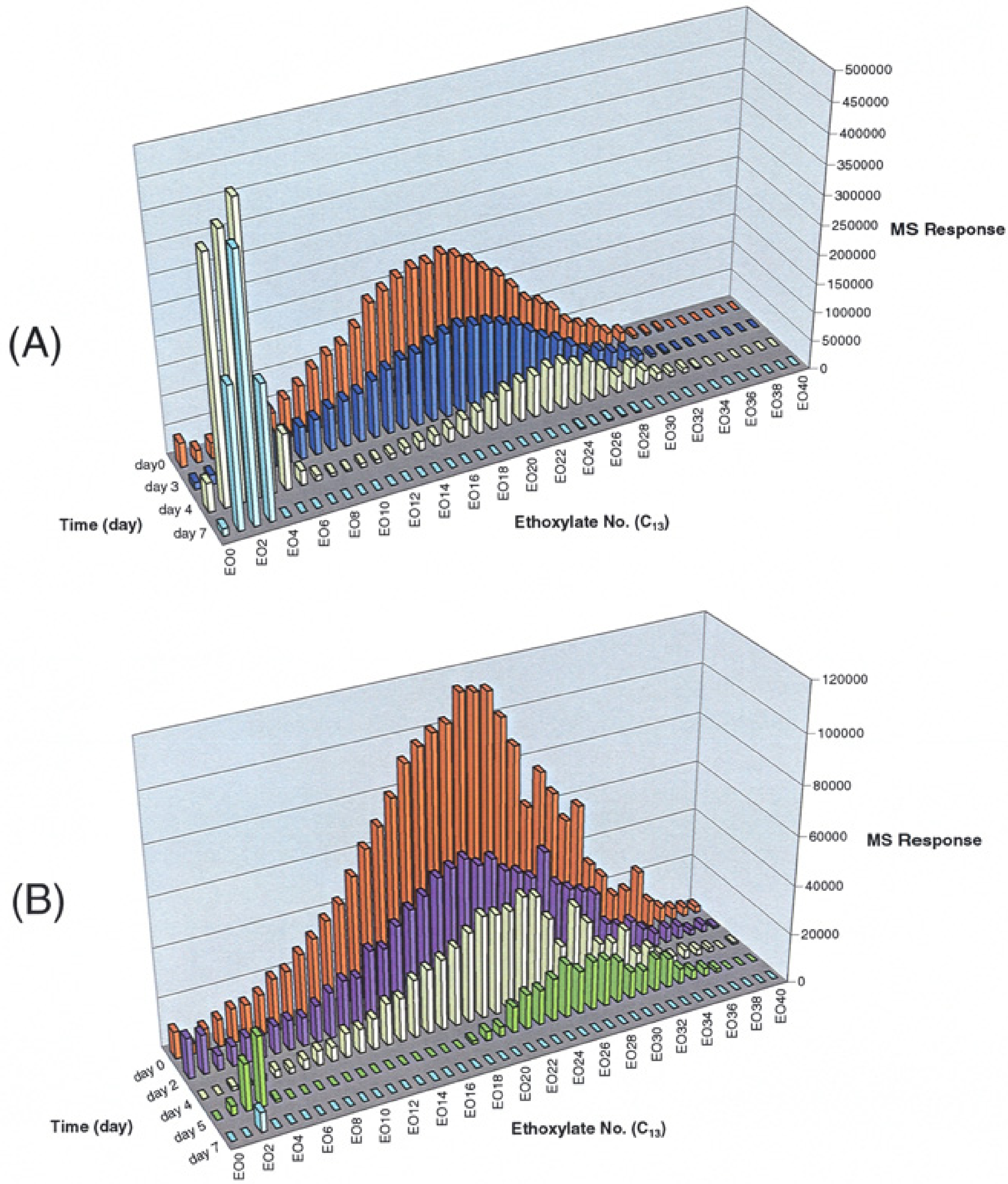
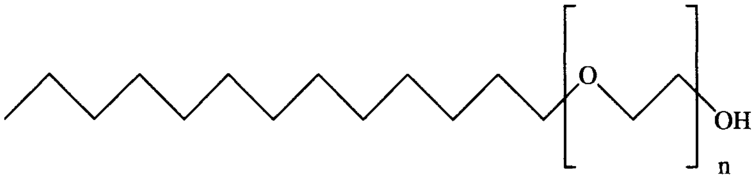
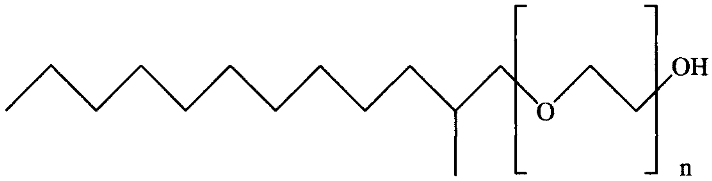


Figure 9.4 Typical structures of Lutensol AO30, demonstrating linear "oxo" structure showing (A) H (linear), (B) methyl and (C) ethyl, branching in the β position.

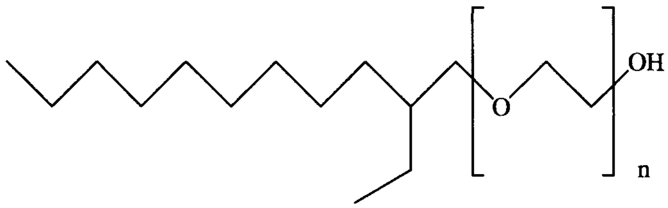
(A)



(B)



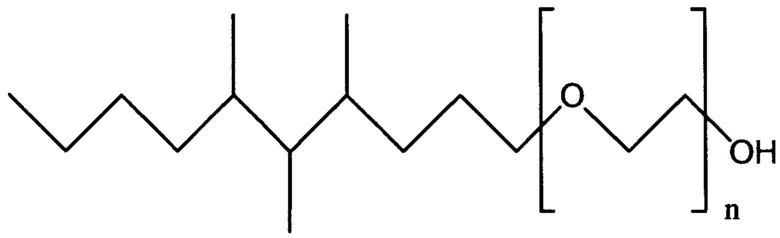
(B)

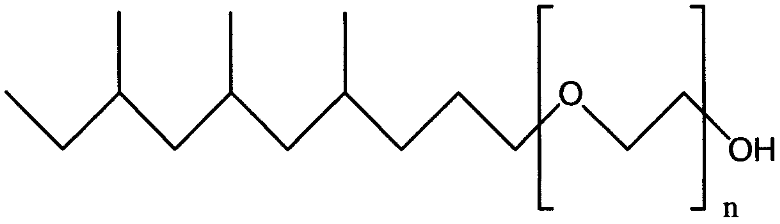


($n_{av} = 23$)

carboxylic acid on the hydrophobe end of the molecule [3]. Formation of free alcohol was not seen in this work. The presence or absence of carboxylic acid would need confirming by future work.

Figure 9.5. Typical structures of Lutensol TO20 (2 to 3 methyl branches per chain)





$(n_{av} = 18)$

SECTION B- BIODEGRADATION OF LUTENSOL AO30 IN A SEALED VESSEL TEST: INVESTIGATION OF METABOLITES

9.4 Introduction

Previous work has shown the biodegradation of Lutensol AO30 in a sealed vessel test was rapid with complete removal of "parent AE" wthin the first 7 days of the study. From this work however the mechanism of biodegradation of the material was not clear. The analysis for loss of parent AE was repeated for consistency with the original work, and at the same time the formation and disappearance of other species needed to be assessed. These include PEG and C_{13} and C_{15} carboxylic acids. As well as specifically looking for such components the data will be fully investigated for the presence of other known intermediates in AE biodegradation (see Figure 2.2). As the analysis for AEs was optimised for hydrophobic component recovery by the addition of 40 % v/v methanol to the sample before passing through SPE, a duplicate sample was analysed to allow the more polar species such as PEG to be extracted from solution. Extraction of carboxylic acids and PEG from test media at pH 3 on a C_{18} SPE cartridge and subsequent analysis by LC/ESI-MS was performed. This extract was also screened in both positive and negative ESI for the presence of any other metabolites.

9.5 *Experimental*

9.5.1 *CHEMICALS AND REAGENTS*

AE analysis was carried out as in Section 9.1, using chemicals and reagents as described earlier. For the assessment of metabolites the following were used: PEG average molecular weight 1450 (PEG 1450), PEG average molecular weight 1000 (PEG 1000), PEG average molecular weight 400 (PEG 400) and PEG average molecular weight 200 (PEG 200) were all obtained from Sigma (Poole, UK). Pentadecanoic acid, 99+ % purity, was supplied by Aldrich (Poole, UK). Tridecanoic acid, 99.7+% purity, was supplied by Fluka (Poole, UK). 1 M HCl for pH adjustment was obtained from VWR (Lutterworth, UK).

A stock consisting of 4000 μg/mL of total PEG (1000 μg/ml mix of each of PEG 200, PEG 400, PEG 1000 and PEG 1450) was prepared in methanol. A stock containing tridecanoic and pentadecanoic acids both at concentrations of 100 μg/mL was also prepared in methanol.

9.5.2 *EXTRACTION PROCEDURES*

Duplicate samples at each timepoint were taken, one extracted for AEs the other for PEG and carboxylic acids. In the following discussion these are referred to as the AE sample and PEG and acids sample, respectively. Samples containing 100 mL of test item were analysed on day 0 to 7 of the study. Control samples, consisting of test media without addition of test material, were also received on the same days. The AE sample was transferred to a 500 mL bottle, the sample container washed with 66 mL of methanol and combined portions taken through the automated SPE procedure on the Zymark Autotrace, extracted on a C_8 cartridge and subsequently derivatised and analysed for total AE by methodology described earlier. The PEG and acids sample was taken to pH 3 by the addition of 1 mL of 1 M HCl and taken through the same Zymark Autotrace procedure but this time loading on to a C_{18} 1 g/6 mL cartridge from Isolute. The final extract was concentrated to less than 1 mL under a gentle stream of nitrogen and transferred to a 1 mL volumetric flask and made up to volume in methanol.

SPE recovery check samples of PEG (4 mg/L total) and carboxylic acid (1 mg/L each component) were also prepared by adding 100 μL and 1 mL respectively of the stock solutions into separate 100 mL aliquots of control media. These were taken through the PEG and acids procedure described above. SPE recovery for AE was checked as described earlier.

9.5.3 LC/MS ANALYSIS

Both extracts were analysed on an 1100 LC/MS G1946B version MSD from Agilent Technologies Ltd. (Stockport, UK). The AE extract was analysed as described earlier (Section 9.2.2), with the exception that the column used was a Luna C_{18}(2) 15cm x 2.1 mm i.d. and 2 mm i.d. C_{18} guard cartridge, both from Phenomenex (Macclesfield, UK).

The PEG and acids sample was analysed for the different components as follows. PEG was analysed using reversed phase gradient LC. The mobile phase consisted of a mixture of A, 5 mM ammonium formate pH 3.0 and B, acetonitrile. The mobile phase composition was 20 % B at the beginning of the gradient and then linearly increased to 100 % B in 20 min. It was then held for a further 10 min before re-equilibrating at the initial conditions for 20 min. The PEG oligomers were separated on the column described above, at a flow rate of 0.20 mL/min and

10 μL of the methanol extracts were injected onto the column, which was thermostatically held at 30 °C.

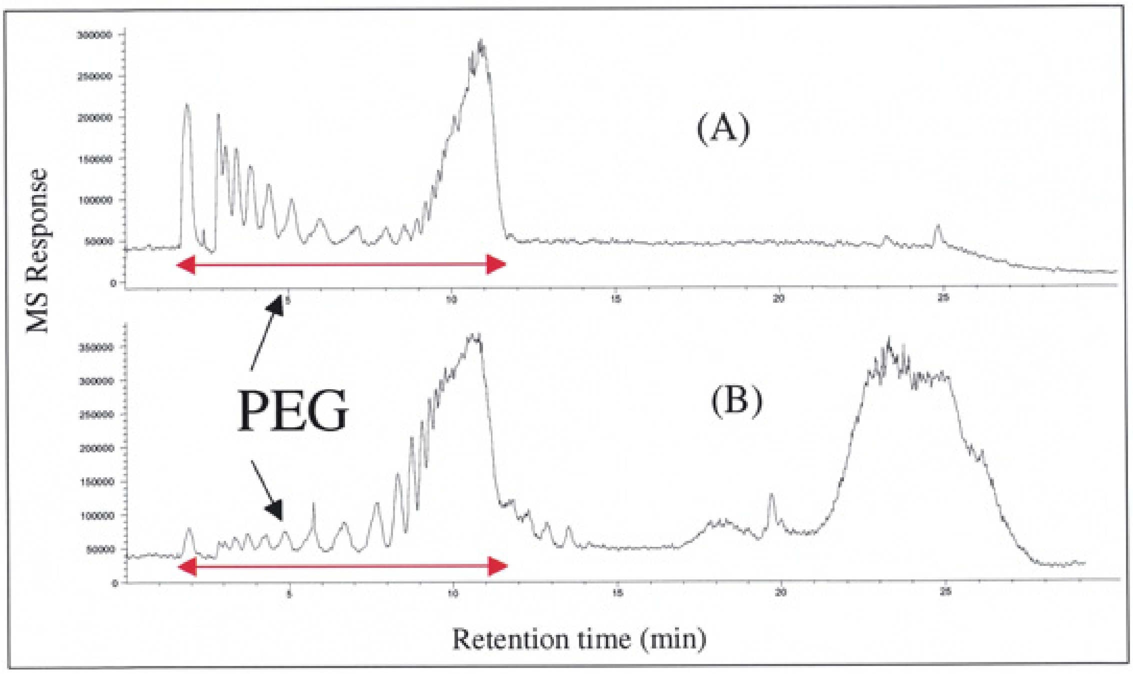
The MS was operated in positive ion mode electrospray with gas temperature 275 °C, drying gas 7.0 L/min, nebuliser gas pressure 35 psi, and capillary voltage 3000 V. The MS was operated in full scan mode over the range *m/z* 120 to 2000. The fragmentor was optimised at 20 V for the PEG analysis. The samples were rerun using the same conditions as above but with a higher fragmentor voltage of 70 V to look for other intermediates. Calibrations standards of 0, 200, 400, 800 and 2,000 μg/mL total PEG were run, along with standard checks at 400 μg/mL to assess MS response throughout the run.

Carboxylic acids were analysed using the same negative ion LC/MS method as for the AE analysis. The MS was scanned from 150 to 300 *m/z* to analyse for tridecanoic and pentadecanoic acids specifically. No diversion of flow was necessary for the MS acquisition and an injection volume of 10 μL was used for this work. The range was increased to 150 to 2150 *m/z* to look for other negative ion species present. The fragmentor was operated at 70 V for both scan range acquisitions. Each acid was analysed over the calibration range 0, 1, 10, 50 and 100 μg/mL.

9.6 Results and Discussion

PEG standards ranging from 0 to 800 μg/mL of total PEG were integrated to give a total area. An example of this is included in Figure 9.6. PEG is seen eluting as a series of peaks from approximately 2 to 12 min. The calibration curve produced from this analysis gave a well-defined quadratic fit ($R^2 = 0.9993$). At the levels detected it was considered that this curve gave a good indication of the amount of PEG in the samples.

Figure 9.6. PEG retention time window in (A) Standard containing 400 μg/mL of total PEG and (B) extract from Day 2 biodegradation sample. Analysis in both cases is positive ESI with the fragmentor at 20 V



SPE recovery samples analysed at 4 mg/L of total PEG in the control media gave a mean recovery of 79.7 (83.3, 76.0), where the results are quoted as mean % (individual replicates), n = 2. A typical mass spectrum of the PEG is also included in Figure 9.7. The PEG can be seen to ionise under the conditions used forming ions $[OH(CH_2CH_2O)_nH + NH_4]^+$ as seen by the series of ions at *m/z* 1048.6, 1092.6, 1136.6…1664.8 (where n = 23, 24, 25……37), respectively. The series of ions are separated by the characteristic Δ *m/z* of 44. The PEG also ionises forming multicharged species. A series of ions resulting from doubly charged PEG (Δ *m/z* 22) and triply charged (Δ *m/z* 14.66) are both evident in the mass spectrum.

The mass spectrum of the PEG released from the AE biodegradation gave rise to a similar TIC to that of the PEG mixture made up from four commercially available molecular weight ranges. The amount of PEG in the sample could be determined by measuring the total area of the TIC in the retention time window (2 to 12 min.) and calculating concentration from the quadratic equation of the curve. Results of PEG determination are presented in Table 9.3. These results are shown alongside the total AE results and amount of AE removed. The disappearance of AE followed a similar pattern to that obtained previously for Lutensol AO30. In this case however quantitation of AE was achieved using a quadratic curve over the calibration range. Mean recovery of Lutensol AO30 using the SPE procedure from the 20 mg/L control samples was 101 % (101, 101) (individual replicates, n = 2).

Figure 9.7 Typical mass spectrum from approximately 10 to 11.5 min of the TICs for (A) a standard and (B) a Day 2 sample, both in positive ESI, fragmentor 20 V.

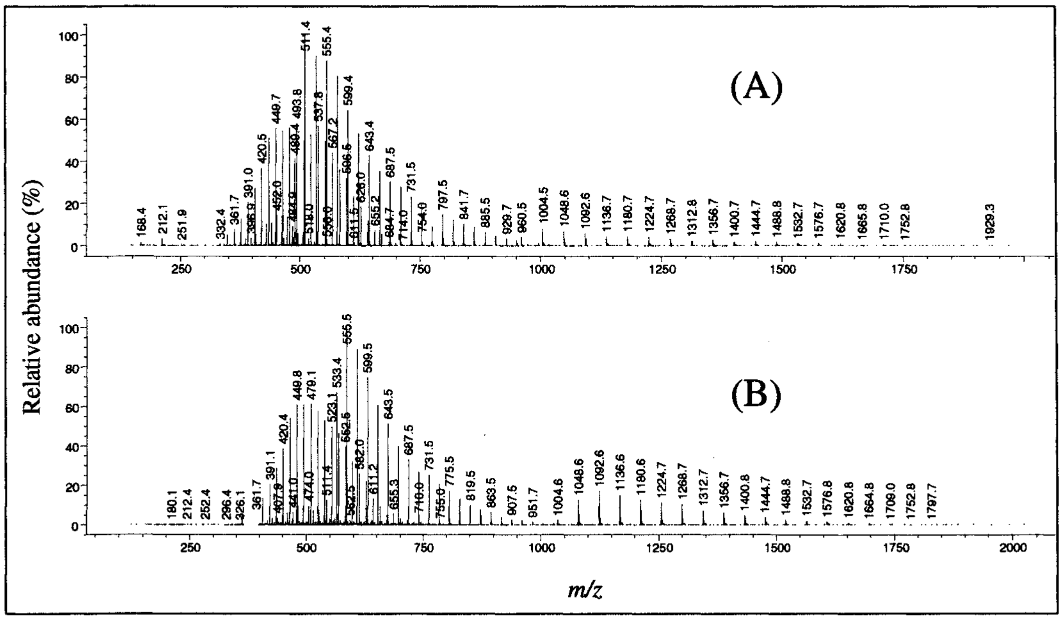


Table 9.3 Concentration of PEG and AE measured in test vessels (mg/L) with biodegraded AE (mg/L) calculated by difference.

DAY	PEG (mg/L)	AE (mg/L)	BIODEGRADED AE (mg/L)
0	<0.1	14.8	0
1	0.3	14.6	0.2
2	5.8	6.3	8.5
3	6.4	3.0	11.8
4	3.1	1.3	13.5
5	0.3	<0.2	14.8
6	<0.1	<0.2	14.8
7	<0.1	<0.2	14.8

An evaluation of the species present in day 2 samples was made in an attempt to postulate the initial mechanism of biodegradation. As can be seen in Table 9.3, AE (8.5 mg/L) has biodegraded to form PEG (5.8 mg/L). Based on the assumption that the average structure of Lutensol AO30 is as shown in Equation 9.1, a central

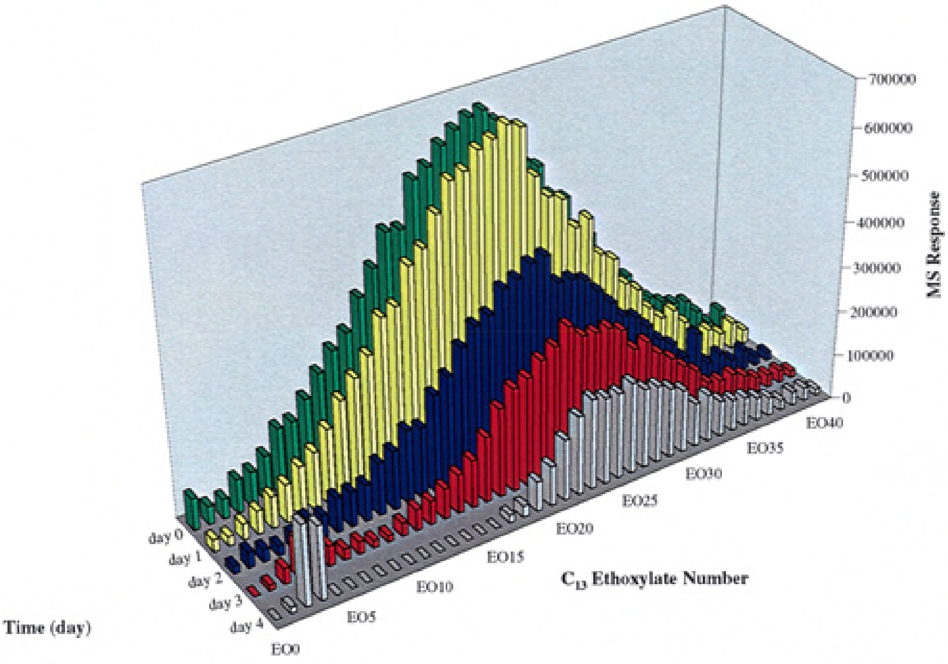
Equation 9.1

$$\underset{1247\ \text{g/mol}}{C_{13}H_{27}O(CH_2CH_2O)_{23}H} \longrightarrow \underset{1029\ \text{g/mol}}{OH(CH_2CH_2O)_{23}H} + \underset{200\ \text{g/mol}}{C_{13}H_{27}OH}$$

fission mechanism would result in release of PEG as a majority species on a weight basis. In Equation 9.1 it can be seen that 83% w/w of PEG would be formed (1029/1247). The large amount of PEG observed in this experiment (68% w/w) appears consistent with this theoretical degradation pathway and implicates central fission as the main mechanism of biodegradation.

Analysis of the extract for carboxylic acids showed the mean % recovery (individual replicates), n = 2 after SPE clean up was 93.6 % (97.1, 84.5) and 85.2 % (86.9, 83.4) for tridecanoic and pentadecanoic acid, respectively. However no tridecanoic or pentadecanoic acids were detected in the real samples with results of < 0.01 mg/L for each acid in samples analysed from day 0 to 7. This result was unexpected as the PEG data suggested central fission had occurred, but neither the alcohol nor the carboxylic acid were detected at sufficient concentration to confirm this. Figure 9.8 shows AE profiles during the course of the study, which is analagous to that seen in the first study and indicates no significant increase in the amount of free alcohol ($C_{13}EO_0$). The absence of alcohols and acids has also been observed by other workers [4], who suggest that biodegradation of the alkyl chain, once released, proceeds more rapidly than the PEG with no detection of metabolites from this part of the molecule.

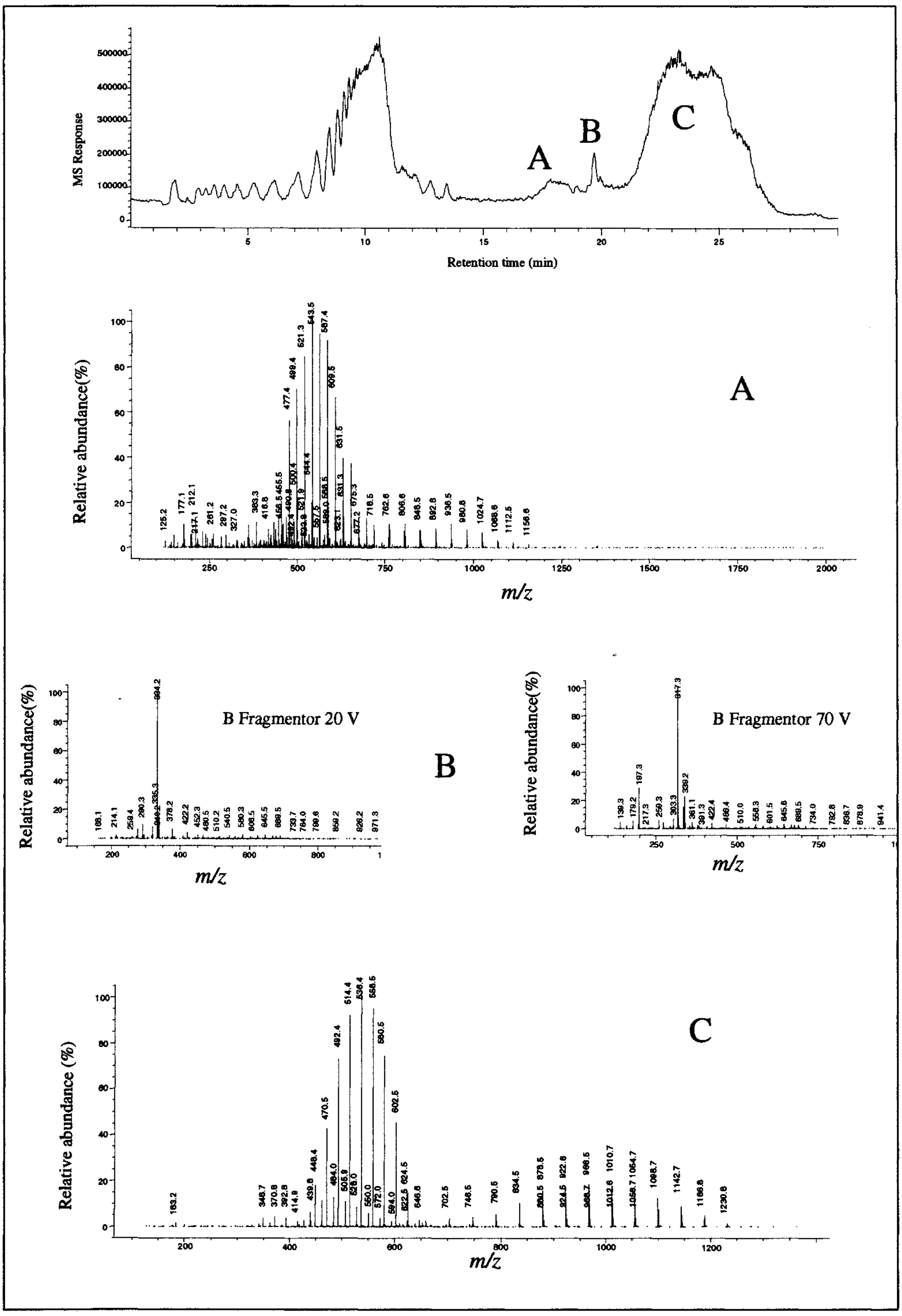
Figure 9.8 AE biodegradation profile from day 0 to 4.



In addition to the PEG, which has been discussed earlier, other metabolites observed in the positive ion ESI data were tentatively identified from their mass spectra. Figure 9.9 shows the TIC at day 2 analysed in positive ionisation mode. Spectra, taken at points indicated on the TIC, are assigned to species listed in Table 9.4. Under the conditions used ammonium adducts are primarily formed. Table 9.4 shows theoretical *m/z* values for the structures identified from the singly charged ions. Figure 9.9 A shows a mass spectrum that can be assigned to two different structures in Table 9.4, one being a product of ω-glycol oxidation and the other the product of ω-alkyl oxidation. This emphasises the care that has to be taken with identification based on unit mass resolution of molecular ions, as given by a quadrupole MS. Either tandem mass spectrometry (MS/MS) to give structural information or time-of-flight (TOF) mass spectrometry to give accurate mass would be required to give more confidence in the identification made. The peak eluting just before 20 min (Figure 9.9 B), has mass spectra displayed at two different fragmentor voltages. At 20 V formation of the ammonium adduct was favoured, whereas at 70 V an $[M+H]^+$ adduct was formed. These ions were identified by a difference in *m/z* of 17 of the two adducts. The peak has been tentatively identified

Figure 9.9. Spectra of main peaks present in TIC of Day 2 sample (positive ion mode ESI, fragmentor 70 V unless stated)

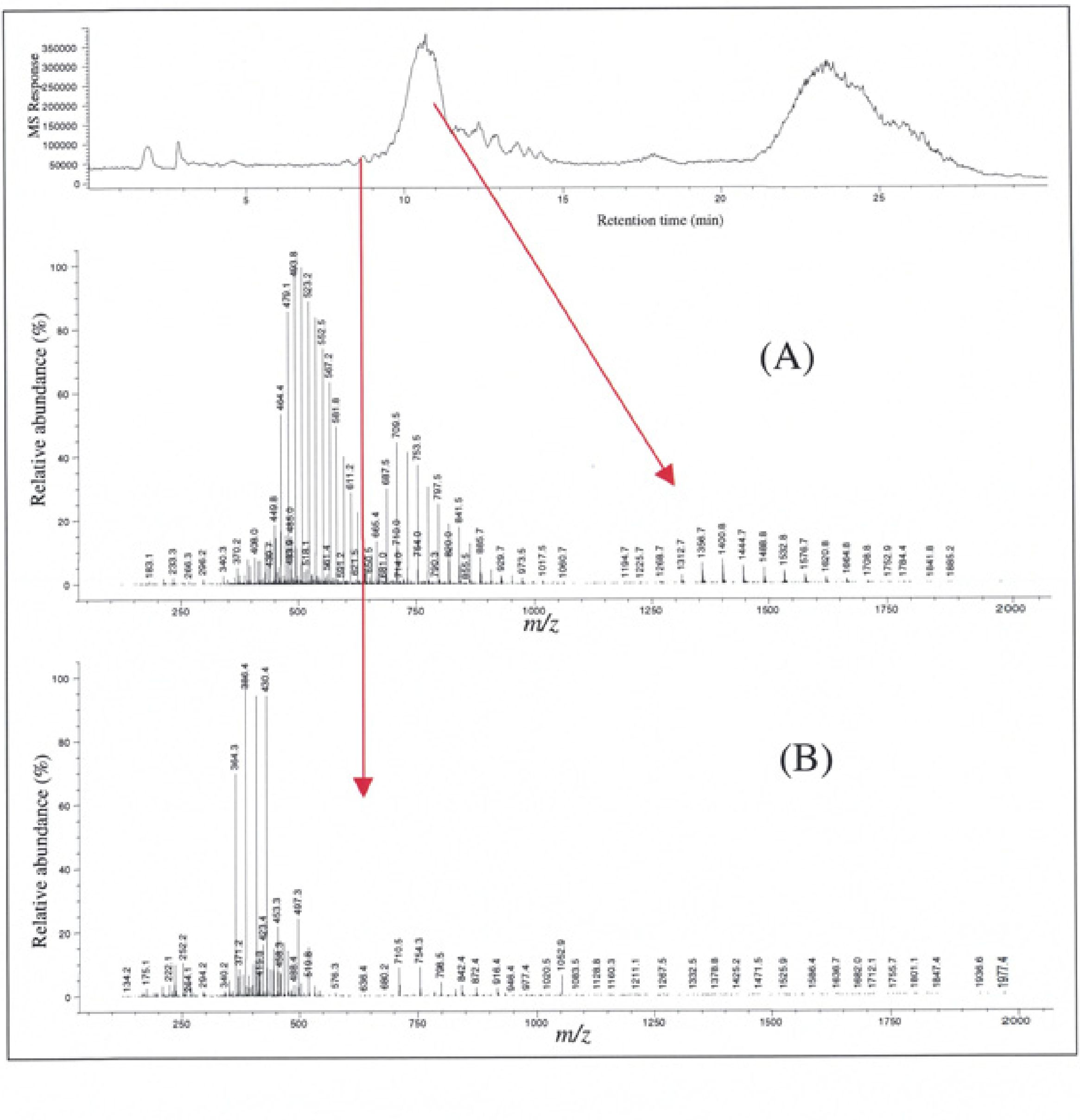
Experimental conditions- Luna C_{18} (2) 150 x 2.0 mm i.d. 5 μm column, flow 0.20 mL/min, mobile phase A, 5 mM ammonium formate pH 3.0 and B, ACN. Gradient 20 % to 100 % B in 20 min, 10 μL injection.



in Table 9.4 as the ω-glycol/ω-alkyl oxidation product. In positive ESI mode, spectra corresponding to unaltered AE ionised as ammonium adducts can clearly be seen (Figure 9.9 C). The PEG was also found to rapidly disappear as shown in Table 9.3. The intermediates involved in the biodegradation of PEG were investigated from mass spectra in the TIC at day 4 (Figure 9.10).

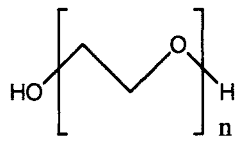
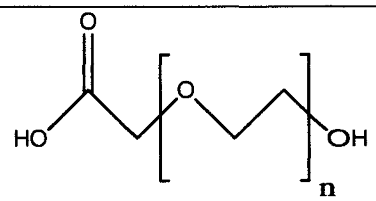
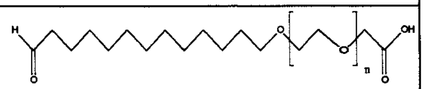
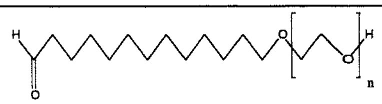
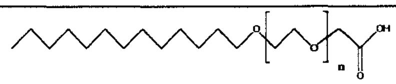
Figure 9.10. Biodegradation of PEG at Day 4 in positive ion mode ESI, fragmentor 20 V.

Experimental conditions- Luna C_{18} (2) 150 x 2.0 mm id 5 μm column, flow 0.20 mL/min, mobile phase A, 5 mM ammonium formate pH 3.0 and B, ACN. Gradient 20 % to 100 % B in 20 min, 10 μL injection.



The presence of both neutral (Figure 9.10 A) and carboxylated PEG (Figure 9.10 B) are confirmed by comparison with the mass spectral assignments in Table 9.4. The structure of the neutral and carboxylated PEG are also shown in the table.

Table 9.4 Structures and diagnostic ions of metabolites formed

METABOLITE	**DIAGNOSTIC IONS** (theoretical masses of ions observed)	**STRUCTURE**
Neutral PEG	$[M + NH_4]^+$ n = 22 to 38 *m/z* 1004.6...........1709.0	
Carboxylated PEG	$[M + NH_4]^+$ n = 14 to 17 *m/z* 710.4, 754.4, 798.5, 842.5	
ω-glycol and ω-alkyl oxidation	$[M + NH_4]^+$ n = 0 to 3 *m/z* 290.2, 334.3, 378.3, 422.3	
ω-alkyl oxidation	$[M + NH_4]^+$ n = 14 to 21 *m/z* 848.6, 892.6, 936.6,...... 1156.8	
ω-glycol oxidation	$[M + NH_4]^+$ n = 13 to 20 *m/z* 848.6, 892.6, 936.6,........156.8	

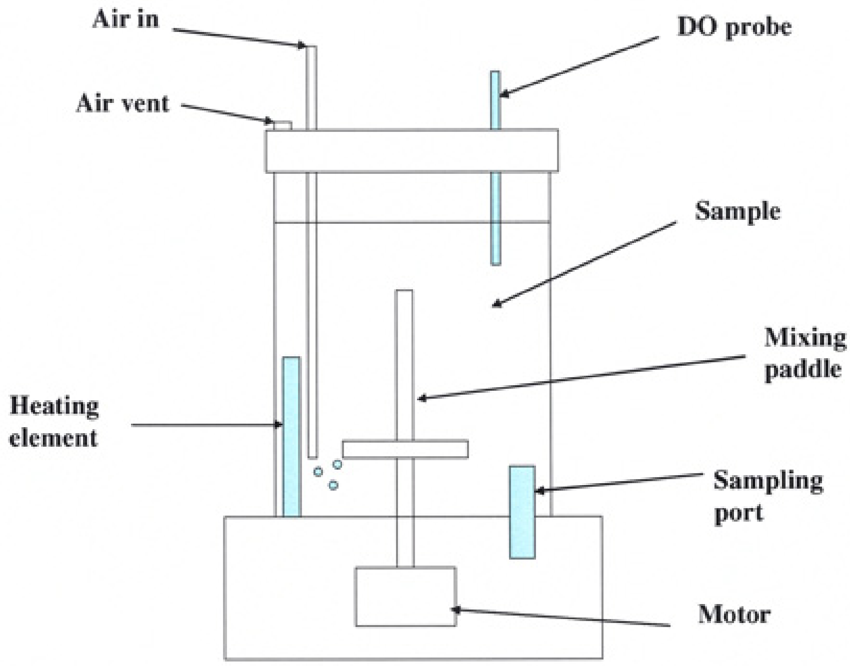
Analysis by LC/MS of Lutensol AO30 in biodegradation samples showed PEG in the approximate quantities and oligomeric distribution for a central fission driven biodegradation mechanism to be dominant. The PEG was itself biodegraded by day 6 of the study, with evidence of formation of both neutral and carboxylated PEG as further intermediates. The associated alcohol and/or carboxylic acid, that are thought to be formed from this fission, were not detected. Other intermediates have also been tentatively identified indicating ω-glycol/ω-alkyl oxidation products were also being formed. It is possible that the molecule is attacked at several sites/positions at the same time as central cleavage occurs, which would explain the absence of any intact alkyl chain fragments. This data and data found in the literature [5], would suggest that multiple pathways of biodegradation are likely to be occurring simultaneously during the biodegradation of AEs.

SECTION C- BIODEGRADATION OF NATIVE ALCOHOL ETHOXYLATES IN SIMULATED "DIRECT DISCHARGE" CONDITIONS

9.7 Introduction

In a direct discharge scenario the traditional approach to risk assessment (predicted environmental concentration) / predicted no-effect concentration ratio) for an ingredient to determine a safety margin becomes meaningless due to the large presence of organic materials from sewage. The accompanying increase in biological oxygen demand (BOD), increased levels of ammonia and suspended solids, as well as a depression in dissolved oxygen levels means the presence of detergent ingredients to already poor conditions is likely to have little effect. However, the relative rates of biodegradation of detergent ingredients in comparison to the general organic loading and ammonia present in sewage can be estimated to see if detergent ingredients are removed prior to self purification processes being completed. A simple batch 'die-away' system (Figure 9.11) was explored to determine the rates of primary biodegradation of native levels of AE homologues in a settled sewage sample diluted with river water in comparison to the chemical oxygen demand (COD) and ammonia present.

Figure 9.11 Batch 'die-away' system



9.8 Experimental

9.8.1 BIODEGRADATION STUDIES

A batch reactor system was prepared and dosed with 2.6 L of a settled sewage / river water mixture (dilution factor =3, 1 part settled sewage to 2 parts river water), simulating a heavily polluted system and worst case scenario for direct discharge. Settled sewage was collected from Broadholme STW, treating predominately domestic waste (% trade flow = 6.2 and % trade organic load = 12.4). River water was sampled from the River Great Ouse, Felmersham Bridge, Felmersham, Bedfordshire. The test system was operated for a five-day period and the temperature was controlled at 20 °C. A slow stream of air was continually pumped through the test system to ensure the test media was saturated with dissolved oxygen (DO). The test system was simulating an environment of continual re-aeration and a mixing speed of 125 rpm was maintained throughout the duration of the test. Periodically samples were removed and analysed for chemical oxygen demand (COD) and NH_4 over the 5 day period. Samples (30 mL) were also provided for the analysis of AEs.

9.8.2 CHEMICAL ANALYSIS

AEs methodology

Samples were analysed according to procedures in Section 5.1.1 – 5.1.3, for chemicals and reagents, commercial samples used as standards and standard preparation, respectively. SPE extraction (Section 5.3.1), derivatisation (Section 5.4.1) and LC/MS (Section 5.5.1) were carried out as described. Any details specific to these samples are described in the following text.

Extraction procedures

30 mL samples of the river water/influent were supplied at 0, 5, 8, 24 hr for analysis. The samples were supplied in a 200 mL bottle, allowing the addition of 66mL of methanol and 70 mL Ultrapure water before taking through the SPE procedure. A quality control (QC) sample prepared at 3mg/l of total AE in Ultrapure water was used to monitor the performance of the method. The same SPE procedure was applied to 100 mL of this solution.

LC/MS analysis

The AE derivatives were separated using a Luna C_{18} 5 µm,15cm x 2.1mm i.d and 2 mm i.d. C_{18} guard cartridge, both from Phenomenex (Macclesfield, UK). For calibration standards the required amount of pyridine stock solution was added to derivatisation solution to give a calibration in the range 0 to 60 µg/mL total AE, using 5 data points.

9.9 Results and Discussion

Extracted ions of the individual ethoxylate derivatives (Table 6.5) and % w/w values of the AEs in the Commercial mix (Table 6.9) were used for quantitation purposes. For each ion linearity in the standards was proven over the range 0 to 60 µg/mL total AE (R^2 typically > 0.99). A 10-fold dilution in derivatisation reagent was necessary to bring the MS response of samples into the calibration range. The QC sample showed recovery of all the individual ethoxylate species (126 in total) to be in the range of 61 to 142 % (with 109 species in the range 80 – 120 %).

Concentrations of $C_{12}EO_{0\text{-}20}$ to $C_{18}EO_{0\text{-}20}$ in 0, 5, 8 and 24hr samples are shown in a graphical representation (Figures 9.12- 9.17). This data shows primary degradation of the majority of the 0hr AE distribution for each homologue after 24hr.

Die away and half-lives

An example of how the data was used in the Environment Section of the Safety and Environmental Assurance Centre is included in the following discussion. The term die way is associated to tests of this nature where surfactants are exposed to micro-organisms in an isolated or batch system. In these studies the concentration of AE at a given time is assumed to follow a first order reaction in equations taken from Swisher [3]:

$$\frac{-dC}{dt} = kC \qquad \text{Equation 9.1}$$

where C is the concentration of surfactant and k is the first order reaction rate constant. In integrated form Equation 9.1 becomes:

$$\text{Ln } C = -kt + \ln C_0 \qquad \text{Equation 9.2}$$

Where C_0 is the concentration at time t = 0.

Substituting $C/C_0 = 0.5$ into Equation 9.2, where the half-life is the time needed for the surfactant to drop to half its initial concentration gives us Equation 9.3:

$$t_{1/2} = \frac{-\ln 0.5}{k} = \frac{0.693}{k} \qquad \text{Equation 9.3}$$

From our data k can be determined by calculating the gradient of a plot of the natural logarithm of concentration against time (Figure 9.18). From this data k for C_{12} EO_0 is determined to be 0.32 hr^{-1} or 7.8 day^{-1}. It is then possible to calculate $t_{1/2}$ as 2.1 hr^{-1} from Equation 9.3.

This data was repeated for the other $C_{12}EOs$ and compared to the COD rate over the course of the study (k_{COD} = 0.32 day^{-1}, $t_{1/2}$ = 51.5 hr) [6]. This led to the following data presented in Table 9.5, which indicated $C_{12}AE$ removal was consistently more rapid than the removal of COD, ranging from 24 to 2.6 times faster depending on EO group. It is evident for C_{12} AEs that the lower EOs are biodegraded more rapidly.

Table 9.5 Decay rates and half-lives for $C_{12}EO_{0-15}$

C_{12} ethoxylate	**k (day^{-1})**	**$t_{1/2}$ (hr^{-1})**	**$k_{(cod)}/k_{(C12)}$**
EO_0	7.8	2.1	24
EO_1	4.2	4.4	12
EO_2	4.4	3.8	14
EO_3	4.2	3.9	13
EO_4	3.8	5.0	12
EO_5	3.3	5.9	8.8
EO_6	3.2	6.0	9.9
EO_7	3.0	5.5	9.3
EO_8	2.5	7.2	7.1
EO_9	2.3	8.0	6.4
EO_{10}	2.0	9.3	5.5
EO_{11}	2.0	9.5	5.4
EO_{12}	1.3	13	3.9
EO_{13}	2.3	20	2.6
EO_{14}	0.91	19	2.7
EO_{15}	0.70	23	2.6

Figure 9.12. Biodegradation of C_{12} alcohol ethoxylates

Conditions- 30 mL test liquors containing sewage influent/river water were prepared by adding 70 mL of water and 66 mL of methanol before extracting on a C_8 1 g Isolute cartridge. The extract was dried, derivatised and analysed by negative ion LC/ESI-MS. The individual ethoxylates were quantified using extracted ions and plotted as shown below.

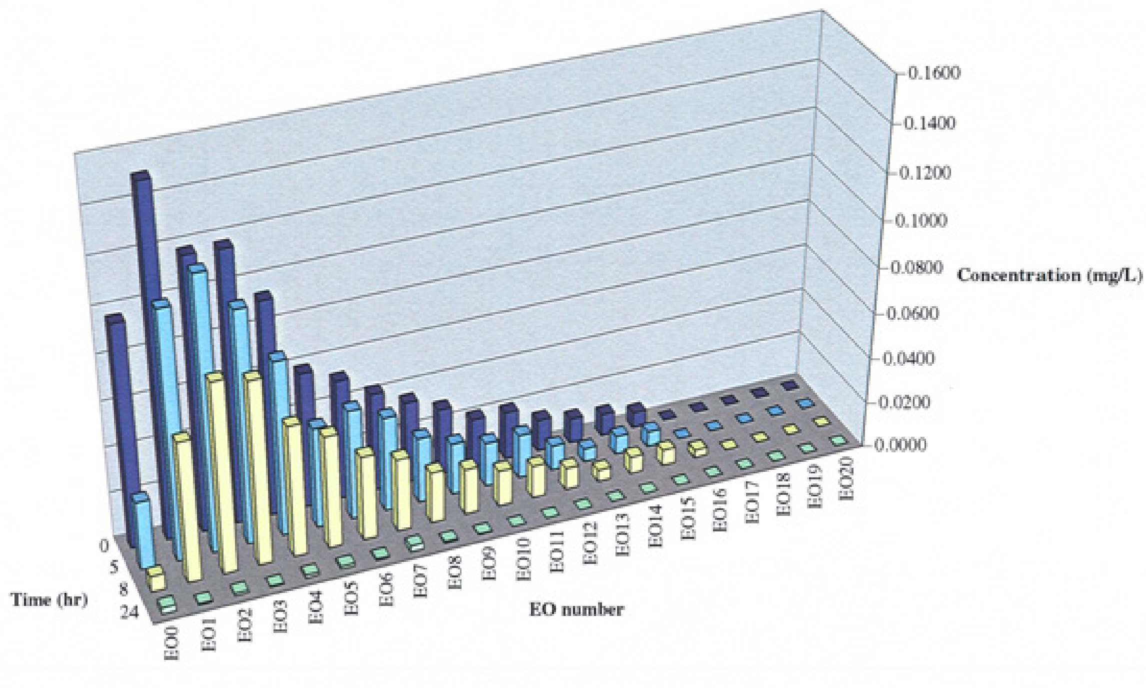


Figure 9.13. Biodegradation of C_{13} alcohol ethoxylates

Conditions- 30 mL test liquors containing sewage influent/river water were prepared by adding 70 mL of water and 66 mL of methanol before extracting on a C_8 1 g Isolute cartridge. The extract was dried, derivatised and analysed by negative ion LC/ESI-MS. The individual ethoxylates were quantified using extracted ions and plotted as shown below.

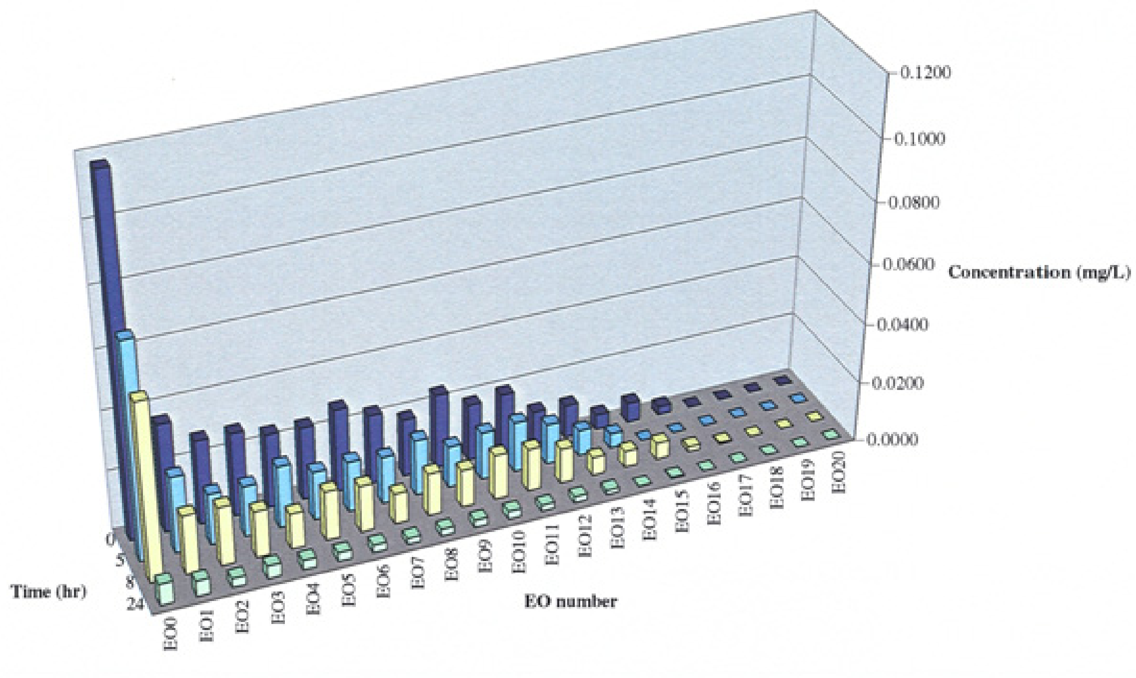


Figure 9.14. Biodegradation of C_{14} alcohol ethoxylates

Conditions- 30 mL test liquors containing sewage influent/river water were prepared by adding 70 mL of water and 66 mL of methanol before extracting on a C_8 1 g Isolute cartridge. The extract was dried, derivatised and analysed by negative ion LC/ESI-MS. The individual ethoxylates were quantified using extracted ions and plotted as shown below.

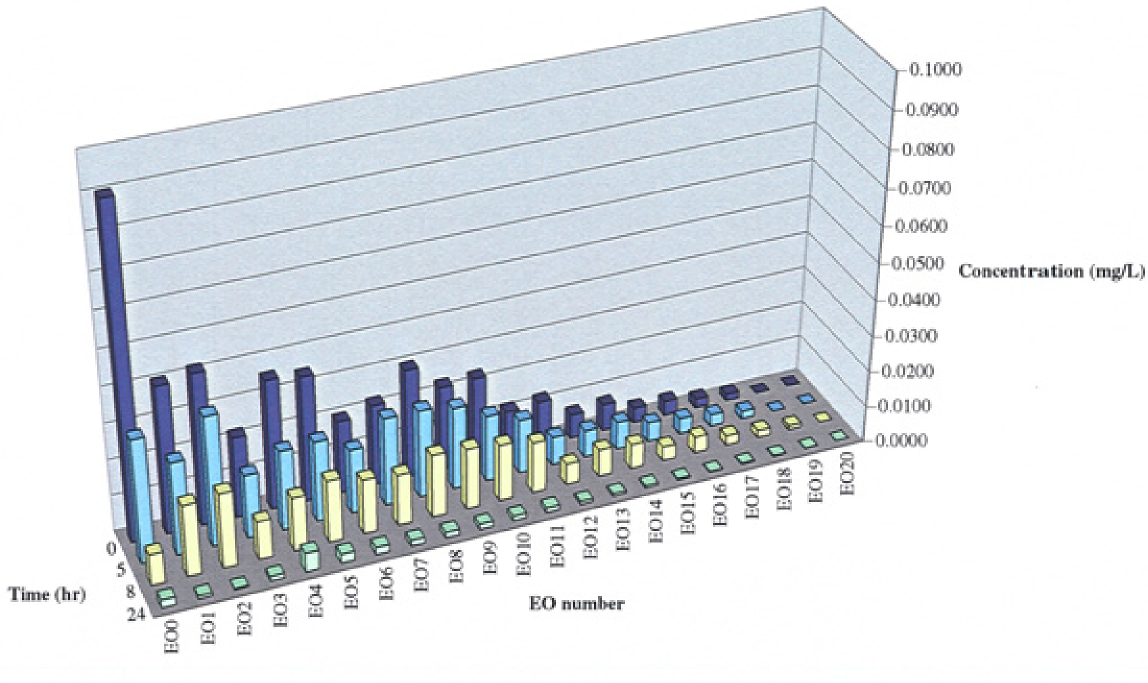


Figure 9.15. Biodegradation of C_{15} alcohol ethoxylates

Conditions- 30 mL test liquors containing sewage influent/river water were prepared by adding 70 mL of water and 66 mL of methanol before extracting on a C_8 1 g Isolute cartridge. The extract was dried, derivatised and analysed by negative ion LC/ESI-MS. The individual ethoxylates were quantified using extracted ions and plotted as shown below.

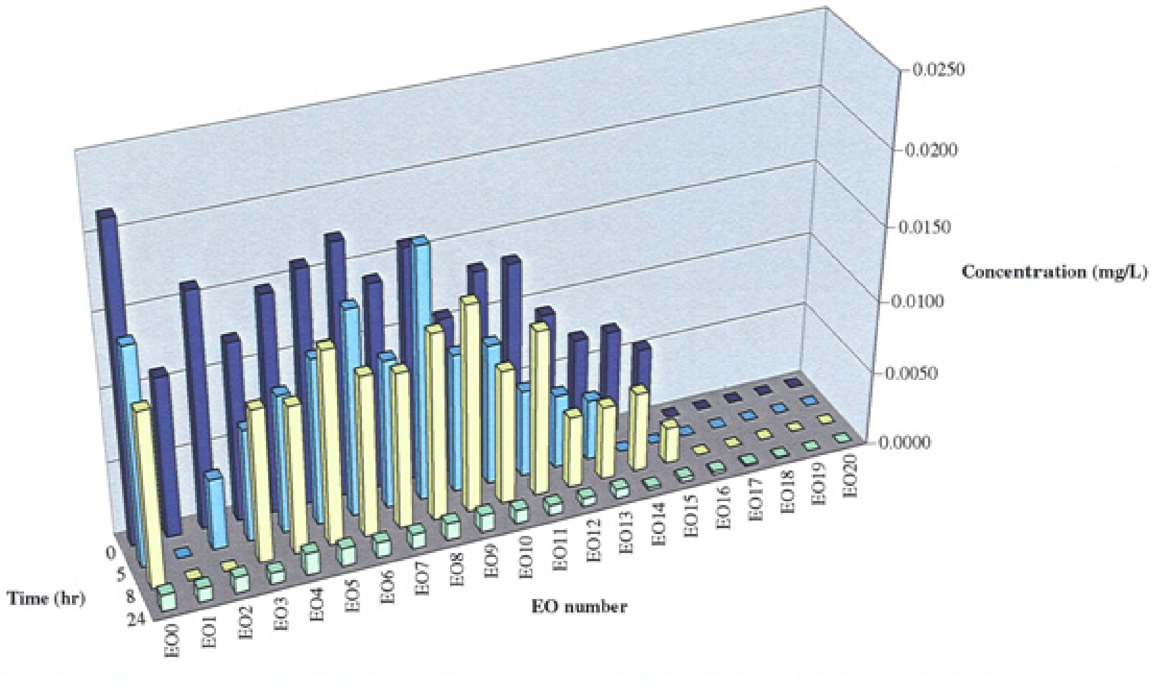


Figure 9.16. Biodegradation of C_{16} alcohol ethoxylates

Conditions- 30 mL test liquors containing sewage influent/river water were prepared by adding 70 mL of water and 66 mL of methanol before extracting on a C_8 1 g Isolute cartridge. The extract was dried, derivatised and analysed by negative ion LC/ESI-MS. The individual ethoxylates were quantified using extracted ions and plotted as shown below.

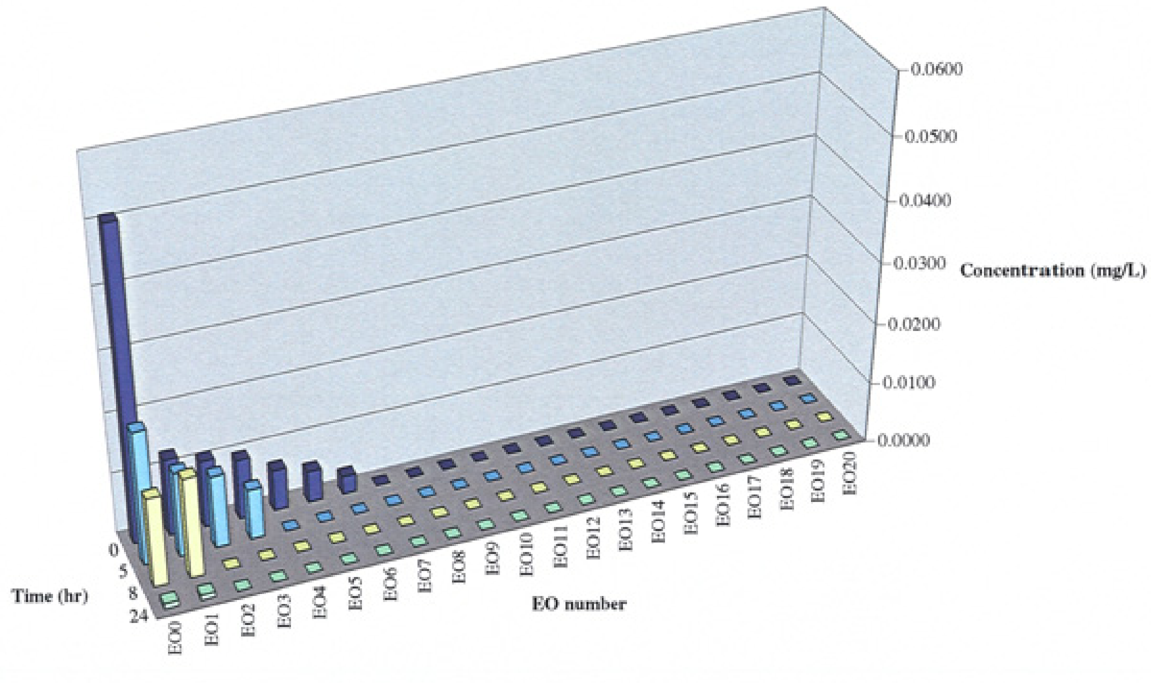


Figure 9.17. Biodegradation of C_{18} alcohol ethoxylates

Conditions- 30 mL test liquors containing sewage influent/river water were prepared by adding 70 mL of water and 66 mL of methanol before extracting on a C_8 1 g Isolute cartridge. The extract was dried, derivatised and analysed by negative ion LC/ESI-MS. The individual ethoxylates were quantified using extracted ions and plotted as shown below.

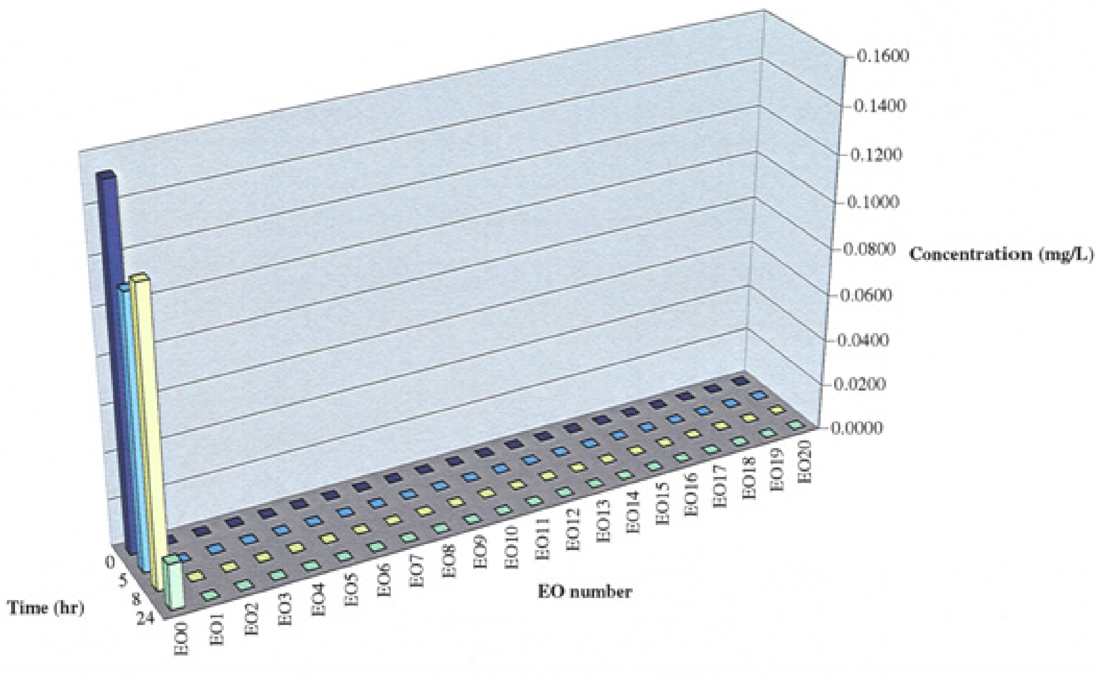
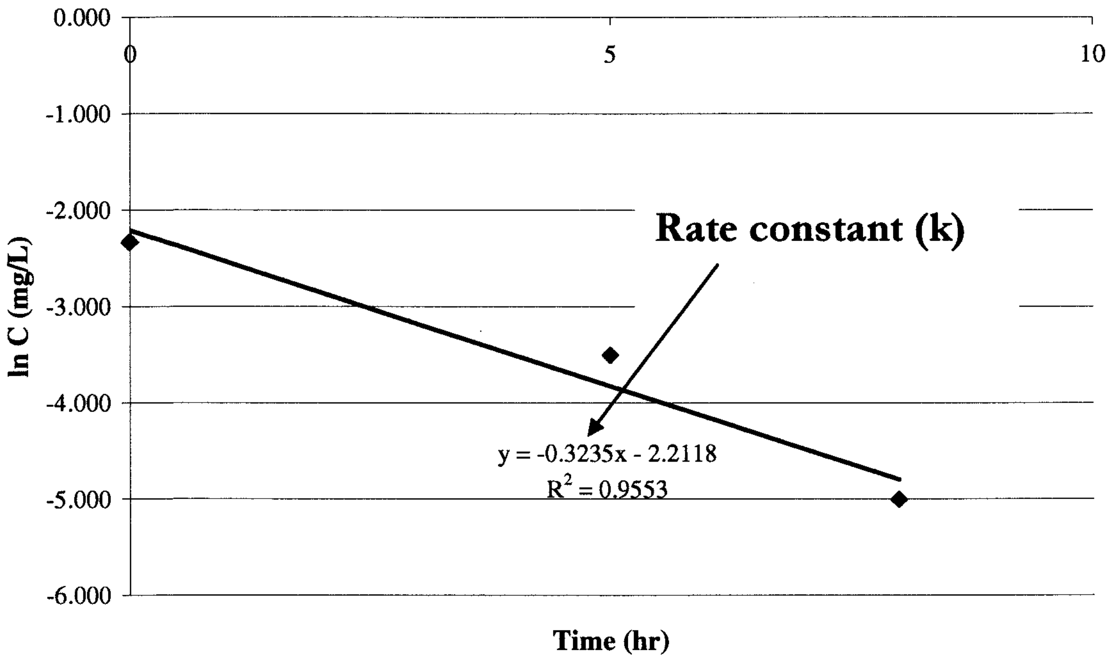


Figure 9.18 Die-away of C12 fatty alcohol ($C_{12}EO_0$)



9.10 Conclusion

Work reported in Part A of the Chapter employed the phthalic anhydride derivatisation LC/MS procedure to study the biodegradation of two long EO-chained AEs. From extracted ions the more linear hydrophobe (AO30) was seen to biodegrade more rapidly and completely than the branched hydrophobe (TO20). A build up of low EOs was seen in TO20, indicating non-oxidative removal of EO groups was occurring.

In Part B biodegradation intermediates of AO30 were also analysed, which confirmed central cleavage as the main biodegradation mechanism for this compound. No residual metabolites were detected. The absence of free alcohol and carboxylic acid indicate either very rapid removal once formed or concurrent attack of the molecule at other sites, preventing the detection of these by-products of central cleavage.

In Part C native AEs were studied under simulated direct discharge conditions. Rapid removal of AEs, quicker than COD removal were shown indicating these surfactants would not have an impact in the environment over and above the general organic loading of a sewage discharge. Decay rates were obtained from the data and generally all native AEs had undergone primary biodegradation within 24 hr.

Reference List

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Chapter 10

CONCLUSIONS AND FURTHER WORK

10.1 Conclusions

Methodology is described capable of determining the "fingerprint" of alcohol ethoxylates (AEs) in aqueous and solid environmental matrices after optimisation of both the mass spectrometry and also the extraction techniques. The entire ethoxylate (EO) range of the Commercial samples tested, containing 126 ethoxymers i.e. $C_{12-18}EO_{0-20}$, was analysed using negative ion electrospray ionisation (ESI) liquid chromatography/mass spectrometry (LC/MS) of phthalic anhydride derivatives. The negative ion formation gave mass spectra free from interference and with good sensitivity. The negative ions formed in this method were free from competition with different adducts and multiple charged spectra that can result in positive ESI of AEs in both derivatised and underivatised form. A balanced molar response of ionisation over the EO range was witnessed, which gave EO distributions comparable with NMR average EO numbers. The benefits of the method were seen in the analysis of environmental samples which indicated significant levels of fatty alcohols (FAs) or EO_0 in activated sludge and final effluent samples, which may have been missed or underestimated with traditional approaches. Analysis of an accurate AE profile in biodegradation studies, where formation of lower EOs was key in mechanistic studies, was also made possible by the method developed.

The phthalic anhydride derivatisation method was optimised using LC/MS as described in Chapter 6. Originally the derivatives were being analysed in positive ion ESI as ammonium adducts. The structure of the derivative, which contains a carboxylic acid group, suggested that negative ion would be favoured. It appeared that direct infusion of the derivative solutions by either flow injection analysis or syringe infusion did not allow negative ion formation. Pre-dilution of a concentrated derivative in mobile phase overcame this problem and allowed optimisation of the method using syringe infusion. The chromatographic method was successfully linked to the LC/MS and miniaturised onto a 2.1 mm id column.

This optimised the ESI response of the pneumatically assisted spray formation and also conserved mobile phase usage.

A solid phase extraction (SPE) method was developed, capable of measuring native levels of AEs in sewage influent and effluent samples, as described in Chapter 7. The aim of this work was to extract the entire EO range from these matrices. This is an area where even current Environmental Risk Assessment and Management (ERASM) monitoring data shows evidence of lower recovery of the more hydrophobic AEs [1]. Generally the more hydrophobic AEs e.g. $C_{18}EO_{0\text{-}10}$ are not recovered by SPE very efficiently. The SPE phase and elution solvents were selected that would elute the entire polarity range of AEs. More critically, however, hydrophobic losses were minimised in the loading of aqueous samples onto cartridges by the addition of 40 % v/v of methanol. This high concentration of methanol was key in increasing the recovery of $C_{18}EO_{0\text{-}10}$ AEs, but at the same time did not incur losses of the more polar AEs e.g. $C_{12}EO_{10\text{-}20}$. Incorporating these optimisations the combined SPE LC/ESI-MS method was capable of analysing AEs, providing more efficient recovery of AEs across the C_{12} to C_{18} range than previously reported in the literature. Recoveries from final effluent spiked at 100 μg/L total AE, for the 126 species analysed, were found to be in the range 55 – 117%, with approximately 100 of the individual analytes having recoveries of 90 – 105 %. A method detection limit (MDL) of 0.02 μg/L for individual ethoxylate components was reported with the instrument operated in scan mode over the range m/z 300 to 1300.

Methodology for extraction of AEs from sediment and sludge samples is described in Chapter 8, in conjunction with the SPE LC/ESI-MS technique already developed. Method development was simplified using a mix of individual EOs, e.g AEs containing EO_0, EO_2, EO_8. However it soon became apparent that this range of EOs, which had been more critical in optimising SPE methodology, was not the key polarity range in solid sample method development. In fact the longer chain EOs were bound more strongly to the sediment by hydrogen-bonding [2] and proved more difficult to extract. Analysis of Commercial AE mixtures showed that increasing Soxhlet times from 6 to 16 hr, using methanol as a solvent, gave recovery of EOs ≤ 17 at approximately 70 % and 90 % for 6 and 60 mg/kg total AE, respectively. The ASE data gave comparable results for the same EO range but gave significantly better recovery for EO_{18-20}. Ultrasonic extraction was also evaluated but appeared to be an even weaker extraction technique than 6 hr Soxhlet (recovery of EOs $\leq$ 14 approximately 50 % and 78 %, respectively at 6 mg/kg of total AE). On optimisation of methodology an MDL was found to be 0.01 mg/kg for each component. This MDL was assessed for 10 g of sediment extracted to a final volume of 1 mL, via an SPE clean-up. The accelerated solvent extraction (ASE) methodology was the preferred technique due to overall extraction efficiency and reduced extraction time (30 min compared to 16 hr by Soxhlet). The ASE methodology was then applied to wet activated sludge samples, which were dried by grinding with hydromatrix. Mean spiked recovery for C_{12}, C_{13}, C_{14}, C_{15}, C_{16} and C_{18} AE homologues of 77, 71, 72, 80, 81 and 82 %, respectively were obtained. Analysis of sludge showed that of the 163 mg/kg of total AEs detected, 93 % w/w of this was accounted for by the C_{12-18} FAs (see Section 10.2)

A study of the biodegradation mechanisms of two long EO chain AEs was carried out and is described in Chapter 9. The two molecules differed in the structure of the hydrophobe which meant different biodegradation mechanisms were expected. By analysing AE profiles during the biodegradation test the SPE LC/ESI-MS method developed allowed mechanisms of EO removal to be assessed. A predominant mechanism in branched AE biodegradation, observed from studying the AE profile in the first week of the study, was non-oxidative loss of EO units and a subsequent build up of lower EO's. For this to occur the molecule was biodegraded from the hydrophile end i.e. hydrophilic/glycol cleavage. The more linear hydrophobe did not show this build up in low EOs. Investigation of metabolites was carried out for the linear AE in biodegradation samples, which showed PEG in the approximate quantities and oligomeric distribution for a central fission driven biodegradation mechanism to be dominant. The PEG was itself biodegraded by day 6 of the study, with evidence of formation of both neutral and carboxylated PEG as further intermediates. The associated alcohol and/or carboxylic acid, thought to be formed from this fission, were not detected. Other intermediates have also been tentatively identified indicating ω-glycol/ω-alkyl oxidation products were also being formed. It is possible that the molecule is attacked at several sites/positions at the same time as central cleavage occurs, which would explain the absence of any alkyl chain fragments. This data and data found in the literature would suggest that multiple pathways of biodegradation are likely to be occuring during the biodegradation of AEs.

The biodegradation of native AEs from sewage influent samples, in a simulated direct discharge scenario, was also studied and described in Chapter 9. These results indicated that the removal of AE was rapid and quicker than the removal of the general organic loading from the sewage. This showed that, on the basis of the laboratory scale tests, AEs would not impact rivers in situations where sewage treatment was absent. Significantly in this study of the biodegradation of AEs present in sewage, there was no build up of FAs.

10.2 Further work

Further work can be divided into two main areas. Firstly a method has been presented which is capable of analysing AEs in environmental samples. The robustness of the method ideally would be tested more rigorously in environmental surveys of both aqueous and solid samples from other locations, containing other detergents, pollutants and endogenous materials. The transferability of the method to other users and instruments and laboratories could also be assessed in this work. A more detailed survey of the presence of AEs at different stages in wastewater treatment plants (WWTPs) could also be carried out to aid risk assessment studies. Data from this thesis has shown the presence of significant levels of FAs, over the levels that might be expected from an AE source, in the samples tested. This is consistent with other recently published monitoring data [1], using the pyridinium derivatives and LC/MS described in Section 3.1. Similar results achieved by a different derivatisation and analysis would support this data, with high FAs being questioned as an artefact of the method. A survey on the distribution of AEs and FAs in the various stages of an activated sludge WWTP, with attention to partitioning between solid and aqueous compartments would be valuable. Similarly analysis of sediments in rivers down stream of WWTP's would determine the final impact of the AEs and FAs in the environment.

Secondly in the biodegradation studies, an investigation of intermediates formed in the biodegradation of a linear oxo AE resulted in several intermediates being tentatively identified. Methods for the analysis of neutral PEG and carboxylic acids were developed which answered certain questions in postulating biodegradation mechanisms. However an SPE procedure that would separate neutral and acidic intermediates [3] may help in identification of species formed. In addition to this, accurate mass determination of some of the compounds postulated would give better conclusions to the mechanistic studies. Time-of-flight mass spectrometric (TOF/MS) techniques would give useful information about elemental compositions which can confirm or rule out potential molecular formulae [4].

Reference List

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APPENDIX

ANCILLARY TRAINING (2001-2005)

May 2001	1100 LC/MSD software operation (Agilent)
June 2001	Systematic interpretation of EI and CI Mass Spectra (UMIST)
October 2001	Interfacing HPLC with MS (Advanza Institute)
July 2002	Quattro LC (triple quadrupole MS) Operator Training (Micromass)
September 2002	Annual meeting British Mass Spectrometry Society, Loughborough University
December 2002	Presentation skills, Keyturn Training
February 2003	Tekmar Purge and Trap Seminar, Anatune
April 2003	Microsoft Powerpoint 97, Vantage Training Ltd.
June 2003	Microsoft Excel 97, Vantage Training Ltd.
June 2003	5th Environmental MS meeting at the Carden Park Hotel, Cheshire – poster presentation "Determination of alcohol ethoxylates in environmental samples by phthalic anhydride derivatisation and LC/ESI/MS" (awarded prize for best poster)
September 2003	Attended 16th International Mass Spectrometry Conference, Edinburgh- poster presentation as above
November 2003	Oral presentation at Agilent LC/MS Users Group, Wessex water. "Determination of Alcohol Ethoxylates by LC-ESI-MS".

January 2004	"Driving Performance" management training, Maximum Performance
January 2005	Scientific publication 1: C. J. Sparham, I.D. Bromilow and J.R.Dean (2005) "SPE/LC/ESI/MS with phthalic anhydride derivatisation for the determination of alcohol ethoxylate surfactants in sewage influent and effluent samples". J. Chrom. A.
September 2005	Annual meeting British Mass Spectrometry Society, York University– poster presentation, "Biodegradation of nonionic alcohol ethoxylates with high EO content: postulation of a biodegradability mechanism and pathway".
September 2005	Oral presentation at work, "Biodegradation of nonionic alcohol ethoxylates with high EO content: postulation of a biodegradability mechanism and pathway".
December 2005	Scientific publication 2: N. Rehman, C. Sparham, J. Melling, J. van Duynhoven and S. Marshall (2005) "Biodegradation of highly ethoxylated nonionic surfactants: Development of LC/MS methods to determine biodegradation intermediates and pathways". Env. Toxicol. and Chem. (submitted).